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γ-Aminobutyric acid (GABA) is a key inhibitory neurotransmitter in the mammalian central nervous system. Two major catagories of receptors, termed $GABA_A$ and $GABA_B$ are activated by the amino acid. Whereas $GABA_A$ receptors appear to be directly involved in synaptic transmission, $GABA_B$ receptors may function as neuromodulatory sites.

Baclofen (BAC), a $GABA_B$ agonist has been shown to have multiple effects on stimulus-evoked increases in second messenger production. For example, BAC augments cAMP formation in the presence of catecholamines but inhibits the response evoked by the direct adenylate cyclase activator, forskolin.

Results from the current study have demonstrated the presence of these $GABA_B$ effects in several mammalian species suggesting a broad physiological relevance. Using a variety of different pharmacophores, evidence is presented supporting the notion that the augmenting and inhibitory efforts of $GABA_B$

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agonists are mediated through pharmacologically distinct reports. Finally, the augmenting response does not appear to be mediated through protein kinase C. However, BAC may facilitate second messenger production by altering the coupling of catecholamine receptor to G-proteins involved in the cAMP cascade.

CONTRACT NUMBER: F-496290-87-C-0071

REGULATION OF NEUROTRANSMITTER RESPONSES IN THE CENTRAL NERVOUS SYSTEM

John Wm. Ferkany, Ph.D.
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February 5, 1990

Final Report for the Primary Contractor for the Grant Period 15 May, 1987 - 14 May, 1989

Prepared for:
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Department of the Air Force
Air Force Office of Scientific Research (AFSC)
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Washington, DC 20332

FINAL TECHNICAL REPORT: F-496290-87-C-0071

Regulation of Neurotransmitter Responses in the Central Nervous System

A. Statement of the work.

As outlined in the initial proposal, substantial evidence is available indicating that γ-aminobutyric acid (GABA), acting through $GABA_B$ receptors, modulates second messenger responses to neurotransmitters in mammalian is central nervous system tissues. In particular, it known that activation of $GABA_B$ receptors augments neurotransmitter-stimulated cAMP formation while inhibiting forskolin-mediated nucleotide production. The contract explored this interaction from a pharmacological and mechanistic perspective. The results of the studies further revealed which component of the cyclic nucleotide generating system is influenced by GABA agonists and the mechanism whereby these drugs alter second messenger responses to neurotransmitters or neuroactive agents. The data are important since they may provide new insights into the modulation of synaptic activity and lead to the development of drugs capable of causing subtle alterations in central nervous system function.

B. Status of Research Effort.

1. Year one period.

Details of research accomplishments made during the initial year of the contract are described in the Annual Technical Report (11-11-88; Exhibit A). First, results of these studies provided suggested that the effects of $GABA_B$ agonists on second messenger production were common to several mammalian species. Second, evidence was provided indicating that pharmacologically distinct $GABA_B$ receptors may mediate the inhibitory and augmenting responses of $GABA_B$ agonists on cAMP production. Third, from a mechanistic perspective, the data suggested that neither adenosine nor the ubiquitous protein kinase C was involved in the $GABA_B$-receptor mediated augmentation of catecholamine-stimulated cAMP production.

The sum of these results were important since they suggested (1) the interaction of $GABA_B$ agonists with neurotransmitter systems was of general physiological relevance to mammalian central nervous system function and (2) that multiple $GABA_B$ receptors may exist in the brain. Although the mechanistic data was negative, by eliminating the involvement of at least one pathway, they served to focus the search for those processes and pathways involved in the observed events.

2. Year two period.

2a. Mechanism. During the second year of the contract, efforts continued to focus on defining the mechanism by which activation of $GABA_B$ receptors influenced cAMP production. Important findings are described in detail in Exhibit B.

Briefly, results of these investigations demonstrated that exposure of brain slices to $GABA_B$ agonists in vitro altered the distribution and affinity of ß-adrenergic receptors in membrane homogenates made from these tissue preparations. In particular, pre-incubation of rat cortical slices in the presence of baclofen (BAC) increased the potency of isoproterenol to inhibit [^{125}I]idopindolol binding to high and low affinity ß-adrenergic receptors. Additionally, the BAC pre-exposure altered the distribution of high and low affinity receptors, significantly increasing the proportion of receptors in the low affinity state. The effect of BAC was stereospecific, and with the pharmacologically important L-isomer being the active component. At low (1 μM) concentrations of L-BAC, the predominant effect was an alteration of receptor distribution; higher (10 μM) amounts of the drug induced shifts in both receptor distribution and receptor affinity.

The endogenous neurotransmitter, GABA (25 μM), mimicked the actions of BAC. Indeed, the influence of GABA was more pronounced than L-BAC and pre-exposure to the amino acid appeared to convert ß-adrenergic receptors to a homogeneous population of sites having an intermediate affinity for isoproterenol. Importantly, the action of GABA was not reduced by the specific $GABA_A$ antagonist, bicuculline, nor did the $GABA_A$ agonist, isoguavacine, mimick the actions of BAC. Finally, the effect of $GABA_B$ receptor activation on ß-adrenergic receptors may be of physiological relevance since an intact tissue preparation was required.

The data were interpreted to suggest that $GABA_B$ receptor activation modified the coupling between ß-adrenergic receptors and guinine nucleotide-binding proteins, and that this modification may, in part, explain the ability of BAC to augment catecholamine-stimulated cAMP accumulation in brain slices.

2b. Pharmacology. Additional studies explored the pharmacological profile of the $GABA_B$ receptor(s) mediating the augmenting and inhibitory effect of BAC on second messenger production. For example, during the first period of the award, data showed that 3-aminopropane phophonic acid (3-APPA), a compound lacking agonist or antagonist properties on catecholamine-stimulated cAMP production, was a moderately potent, BAC-like agonist in the presence of forskolin.

Specifically, during the second period, the effects of the putative $GABA_B$ antagonists 3-hydroxy-saclofen (SAC), 2-butyl GABA and 2-decyl GABA on catecholamine- and forskolin-stimulated cAMP production were examined. Of note, whereas 2-butyl GABA lacked either agonist or antagonist properties and SAC appeared to be a $GABA_B$ antagonist in both systems, 2-decyl GABA preferentially antagonized the augmenting effect of BAC on isoproterenol-stimulated second messenger production. These findings, coupled with the differential effects of 3-APPA on $GABA_B$ receptor-mediated effects lended support to the notion of the multiplicity of $GABA_B$ receptors in brain. Full details of these findings are shown in Exhibit B.

In ancillary studies performed in collaboration with other Nova scientists, but not directly supported by the contract, the effects of $GABA_B$ agonists and antagonists on pulmonary function in vitro was examined. Details of the methods and findings are noted in Exhibit C. Importantly, however, 3-APPA appeared to have $GABA_B$ receptor antagonist properties or tracheal strips, whereas 2-decyl GABA was without effect. Thus, the $GABA_B$ receptor mediating

tracheal strip relaxation may be more similar to central nervous system receptor inhibiting forskolin-stimulated cAMP production.

In sum, these data have been interpreted to support the notion of pharmacologically distinct $GABA_B$ receptors in mammalian tissues. Furthermore, the results predict it may be possible to develop more potent compounds which would discriminate between receptor subtypes thus, subtly modifying synaptic neurotransmitter activity.

2c. Current status. Direct work on the project was terminated at the end of the contract period. An application for additional funding from sources other than AFSC is pending. Further work will be contingent on receipt of such funding.

C. Chronological List of Written Publications.

Scherer, R.W., Karbon, E.W., Ferkany, J.W. and Enna, S.J.: Comparison of baclofen and phorbol esters as augmenters of isoproterenol- stimulated cAMP production in rat brain slices. Soc. Neurosci. Abstracts, 13:1653, 1987 (abs.).

Scherer, R.W., Karbon, E., Ferkany, J.W. and Enna, S.J.: Augmentation of neurotransmitter receptor-stimulated cyclic AMP accumulation in rat brain: differentiation between the effects of baclofen and phorbol esters. Brain Res., 451:361 - 365, 1988.

Scherer, R.W., Ferkany, J.W. and Enna, S.J.: Species-dependent augmentation of receptor-mediated cAMP production by baclofen. Soc. Neurosci. Abs. 14:113, 1988 (abs.).

Scherer, R., Ferkany, J.W., Karbon, E.W. and Enna, S.J.: $GABA_B$ receptor activation modifies beta-adrenergic receptor agonist binding in rat brain cerebral cortex. J. Neurochem., 53:989 - 991, 1989.

Scherer, R.W., Ferkany, J.W. and Enna, S.J.: Evidence for pharmacologically distinct subsets of $GABA_B$ receptors. Brain Res. Bull., 21:429 - 443, 1989.

Karbon, E.W. Zorn, S.H., Newland, R.J. and Enna, S.J.: Pharmacological and biochemical evidence for the existence of multiple $GABA_B$ receptor subpopulations in the central nervous system. First International $GABA_B$ Receptor Symposium, (in press).

D. Key Personnel.

R.W. Scherer, Ph.D., postdoctoral fellow, Nova Pharmaceutical, 1987 - 1989. Direct responsiblity for bench research effort.

J. W. Ferkany, Ph.D., Group Leader, CNS Research, Nova Pharmaceutical, 1984 - present. Oversight of laboratory research.

E.W. Karbon, Ph.D., Research Associate, CNS Research, Nova Pharmaceutical, 1989 - present. Oversight of daily research.

S.J. Enna, Ph.D. Senior Vice President for Scientific Affairs, Nova Pharmaceutical, 1987 - present. Project co-ordination and scientific direction.

E. Coupling Activities.

S.J. Enna, Ph.D., invited speaker, First International $GABA_B$ Receptor Symposium, Cambridge, UK, September 17 - 20, 1989.

REPORT OF THE SUBCONTRACTOR

FINAL PROGRESS REPORT

I. Objectives of Research: (1988-89)

A. To investigate the interactions between brain cyclic nucleotide systems and β-adrenergic, adenosine, and $GABA_B$ receptors.

B. To compare the effects of a variety of selective cyclic nucleotide phosphodiesterase inhibitors for their ability to alter cyclic nucleotide metabolism and/or turnover in brain. These include Rolipram, a centrally active drug that may selectively inhibit brain cyclic nucleotide phosphodiesterase, and Indolidan, a cardiotonic agent that inhibits a cardiac cyclic nucleotide phosphodiesterase associated with the sarcoplasmic reticulum.

C. The development of a cell culture system to probe the interactions between these neurotransmitter systems and specific cyclic nucleotide phosphodiesterase isozymes.

II. General Methodological Approaches:

A. Determine the mechanisms by which pharmacological agents that interact with specific receptors affect the turnover rates of cyclic AMP metabolism in isolated brain slices and cultured PC-12 cells. Adenine-prelabeling is used to label endogenous ATP stores to steady-state levels. Chromatographic methods are used to isolate the amount of cAMP accumulated in the tissue in response to various agonists.

B. Assay of enzyme activities in cell-free preparations, subcellular fractions, and correlate inhibition of drug effects in vitro with their effects in intact brain slice (ex vivo) or cultured cell preparations.

C. Isolation, purification and characterization of specific isozymes of brain cyclic nucleotide phosphodiesterases as an approach to develop more specific probes for examining possible effects of drugs on the cyclic nucleotide phosphodiesterase in brain. The development of specific antibodies to one or more of these isozymes; the use of immunocytochemical methods to localize and/or co-localize the isozymes with receptor populations; and the synthesis of photoaffinity probes and affinity ligands for use in the localization and/or purification of these enzyme systems.

III. Personnel:

A. Dr. Samuel J. Strada devoted ca. 25% of his research effort towards this project, and was compensated at 10% of his salary level.

B. Dr. Robert Garrett, a postdoctoral fellow devoted ca. 80% of his time toward this research project until July 15, 1988. Dr. Garrett then accepted a position as Assistant Professor of Pharmacology at Campbell University.

C. Dr. C-C. Shen spent ca. 80% of his research time (July 15, 1988 - May, 1989) on this project following the departure of Dr. Garrett. Dr. Shen is a research associate in our cyclic nucleotide research laboratories and was familiar with the techniques needed for this project.

D. Mr. Michael Whalin, a graduate student in the Department of Pharmacology devoted a significant portion of his research efforts towards this project in partial fulfillment for his Ph.D. degree in Pharmacology. Mr. Whalin received a stipend from other sources and was not compensated by the grant. Mr. Whalin received his Ph.D. degree and accepted a postdoctoral position at the Georgetown-Fidia Institute for Neurosciences in Washington, D.C.

IV. Significant Findings:

A. The role of cyclic nucleotide phosphodiesterase isozymes in regulating intracellular cAMP levels was studied using rat brain cortical slices as a model system. The rate of cAMP decay in the absence and presence of selective cyclic nucleotide phosphodiesterase inhibitors after stimulation with adenosine or beta-adrenergic receptor agonists was determined using an adenine prelabeling technique.

The studies show that a rolipram-sensitive, high affinity cAMP phosphodiesterase is primarily responsible for cAMP decay in intact cortical slices following elevation of cyclic AMP levels by either adenosine or beta-adrenergic receptor agonists. Interestingly, this isozyme, which is sensitive to inhibition by the drugs rolipram, RO-20-1724 and SQ-65442 contributes only a small perentage of the total cAMP hydrolytic activity measured in cell-free preparations of cortex. This study provides a good example of how data obtained in cell-free preparations do not always reflect effects observed in intact tissue.

A thorough description of these results is provided in an accompanying reprint (Second Messengers and Phosphoproteins 12:311-325, 1989.

B. PC 12 cells were studied as a different model system to explore interactions between receptor systems and/or cyclic nucletoides because: 1) the cells were shown to contain only one isozyme of cyclic nucleotide phosphodiesterase, namely, a Type II (cGMP-activatable) form of cyclic nucleotide phosphodiesterase with 50% of the total activity associated with the particulate fraction; 2) they represent a well-characterized population of rat adrenal medullary pheochromacytoma cells having the same embryonic lineage as brain; 3) the cells in culture respond to nerve growth factor by differentiating from chromaffin-like cells to cells that exhibit a neuronal phenotype; 4) the cell line is well-established and can be maintained in culture yielding large quantities of material for experimentation; 5) cyclic nucleotides have been reported to modulate cellular responsesin PC 12 cells, eg. neurotransmitter release; 6) the cells contain functional adenosine receptors that

are capable of increasing intracellular cAMP levels, and functional atrial peptide (ANF) receptors that lead to increases in intracellular levels of cGMP, and 7) since no cGMP-dependent protein kinase was detected in these cells, functional effects of cGMP may be attributed to interactions with cyclic nucleotide phosphodiesterase rather than cGMP-dependent protein kinase.

cAMP accumulation induced by adenosine in intact cells showed a dose-dependent rise in cAMP levels with peak responses (5-8 fold) at 5 min; removal of adenosine by the addition of adenosine deaminase resulted in the rapid decay of cAMP to basal levels in 3 min. The drugs Papaverine or Trequensin (HL-725), which inhibit the Type II PDE activity in vitro, potentiate adenosine responses, whereas preincubation of PC 12 cells with nitroprusside or the atrial peptide (which increase cGMP levels in these cells), attenuate adenosine-induced cAMP accumulation and increase the rate of decay of cAMP.

We conclude from these studies that increases in cGMP levels regulate cAMP metabolism via the activation of a Type II PDE. These results may have significant implication for the general regulation of synaptic transmission via a dual control mechanism involving both cAMP and cGMP.

A more thorough description of these results is provided in abstract and pre-print accompanying this report.

C. We developed a monoclonal antibody to a Type II isozyme. The antibody was generated against a protein purified from rabbit brain, which had physico-chemical and pharmacological properties very similar to the isozyme identified in PC 12 cells. However, the antibody failed to immunoreact with the enzyme from rat brain or cultured rat PC-12 cells. The antibody, however, did immunoprecipitate activity in rabbit brain extracts and a protein of ca. 110 kD was detected by Western blot analysis. With hindsight, it now seems clear that the development of a polyclonal antibody to the isozyme of interest would have been a more prudent experimental approach.

A comprehensive description of our progress in purifying a membrane-associated enzyme from rabbit brain and the development of specific probes to study this isozyme in brain has been published (Biochim. Biophys. Acta 972:79-94, 1988).

Contract F496290-87-C-0071
Annual Technical Report

Exhibit B

U.S. Air Force Contract (49620-85-K-0014): Subcontract from Noval Pharm. Corp.
Samuel J. Strada, Ph.D., P.I.

Objectives of Research: (1987-88)

1) To examine the role of specific isozymes of cyclic nuclectide phosphodiesterase in regulating cyclic nucleotide metabolism in brain.

2) To explore the interactions between various mediators of synaptic transmission using the cyclic nucleotide system as a probe.

3) To investigate the interactions between beta-adrenergic receptors, adenosine receptors, and peptidergic receptors and the $GABA_B$ system of brain.

4) To determine whether the ability of Baclofen and other $GABA_B$ agonists to potentiate the effects of isoproterenol, norepinephrine and adenosine in elevating cyclic AMP levels in the brain are attributable to specific effects on selective isozymes of cyclic nucleotide phosphodiesterase in brain.

5) To study the actions of rolipram, a relative specific inhibitor of Type IV cyclic nucleotide phosphodiesterase in the brain as compared to the effects of Baclofen.

General Methodological Approaches:

1) Isolate discrete nuclei of brain by punch techniques.

2) Determine activities of cyclic nucleotide phosphodiesterase in these nuclei and in subcellular fractions of brain tissue using methodologies developed to measure selective isoenzymes.

3) Determine the effects of baclofen and other pharmacological agents on the turnover rate of cyclic nucleotide metabolism in isolated brain slices using labeled-adenosine to label endogenous ATP pools and chromatographic methods to isolate the amount of cyclic AMP accumulated in the tissue in response to various pharmacological agents.

4) Correlations of enzyme activities measured in broken-cell preparations with their effects on the ex vivo brain slice preparation.

Personnell:

1) Dr. Samuel J. Strada devoted 10% of his research efforts toward this project

2) Dr. Robert Garrett,Jr., Postdoctoral Fellow, devoted ca. 80% of his research effort toward this project.

3) at various times, Mr. Michael Whalin or Mr. Philip Kithas, graduate students in the Department of Pharmacology worked on various aspect of the study.

Future Directions (1988-89)

1) To continue to explore the mechanism by which $GABA_B$ agonists potentiate the effects of agents that elevate cyclic AMP accumulation in brain with repect to a) the role of guanine nucleotide binding proteins, b) phosphorylation of baclofen-sensitive proteins ,c) cyclic nucleotide phosphodiesterase isozymes.

2) To develop more specific probes to examine the possible effects of drugs on the cyclic nucleotide phosphodiesterase enzyme system in brain in intact tissue. This will include a) development of specific antibodies to each of the isozymes of phosphodiesterase; b) development of immunocytochemical techniques to localize these enzymes to specific neuronal and glial cell populations, and subcellular organelles; c) the development of photoaffinity probes to enable the localization of the enzyme, and its potential covalent alteration in response to pharmacological stimuli.

Project # 1

REGIONAL VARIATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ISOENZYMES IN DISCRETE BRAIN NUCLEI.

Previous studies have shown considerable variations in cyclic AMP and cyclic GMP phosphodiesterase (PDE) hydrolytic activities among different brain regions (Soc. Neurosci. 4:386, 1974). To further extend these observations, we analyzed the activities of three distinct types of (PDE) isoenzymes in homogenates of eight brain regions, including four distinct nuclei in which PDE activity had not previously been investigated. The eight areas include cerebellu (CB), dorsal raphe (DR), hippocampus (HC), locus coeruleus (LC), neocortex (FC), neostriatum (NS), substantia nigra (SN), and ventral tegmentum (VT).

Acutely, opiate treatment has been found to inhibit adenylate cyclase resulting in decreased cAMP levels in neostriatum and cerebral cortex (Tsang et al., Brain Res. 152:521-527, 1978; Law et al., J. Neurochem. 36:1834-1846, 1981). Chronic opiate exposure results in a return of cAMP to control levels, with abrupt withdrawal of opiates associated with cAMP increasing above control levels (Sharma et al., Proc. Natl. Acad. Sci., USA 72:590-594, 1975; Traber et al., Life Sci. 16:1863-1868, 1975; Law et al.). The present study examined not only the brain regional variation and distribution of different types of PDE isozymes, but also examined the same brain areas in rats chronically treated with morphine to ascertain whether development of morphine tolerance was associated with any changes in PDE activity levels.

METHODS:

Male, 150-200 g., Sprague-Dawley rats were killed by decapitation, and the brains rapidly removed into ice-cold, oxygenated, physiological saline, for further dissection. The discrete nuclei were excised as 1mm punches from 0.5 to 0.75 mm thick coronal brain sections. All samples were frozen in liquid nitrogen and kept at -70°C until analysis.

The morphine treated animals were implanted subcutaneously with one morphine pellet (containing 75 mg morphine base) a day for five days. This regimen of chronic morphine treatment has been shown to produce a profound state of tolerance and dependence in rats (J. Blasig et al., Psychopharmacologia 33:19-26, 1973). Control rats underwent identical halothane anesthesia and surgery, but without pellet implantation.

The phosphodiesterase assays were performed using the two step procedure of Thompson et al. (Cyclic Nucl. Res. 10: 62-69). The tissues were homogenized in ice cold buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 5 mM 2-mercaptoethanol, 250 mM sucrose, 20 uM TLCK, 3 mM PMSF, 20 mM benzamidine, and 0.5ug/ml aprotinin. Basal type I activity was assayed using 25 uM cGMP substrate, in the presence of 0.2 mM EGTA. The calmodulin-stimulated activity was assayed with 0.4 mM Ca^{++} and 30 nM calmodulin was purified to homogeneity from bovine brain according to Gopalakrishna and Anderson (Bioc. Biophys. Res. 104:830-836). Cyclic AMP hydrolysis by Type II (cGMP-stimulated) PDE is characteristically enhanced by low levels of cGMP. Type II PDE activity was assayed at 5 uM cAMP substrate in the absence and presence of 2 uM cGMP. The stimulated activity (minus basal) represents the Type II activity present in the sample. Type IV PDE is cAMP-preferring and is often referred to as "high-affinity" or "low-K_m" PDE. The Type IV PDE activity was assayed at 0.25 uM cAMP, in the absence and presence of Rolipram, a specific Type IV PDE inhibitor. The Rolipram-inhibitable activity was considered to represent the Type IV activity present. Protein concentrations were determined using the Bradford dye-binding assay (Analyt. Biochem. 72:248-254), and all PDE activities were expressed as specific activities (nmol/min/mg).

RESULTS:

The SN showed the highest specific activity for all three types of PDE. The LC, DR, VT, and CB displayed very low levels of Types I and II PDE activity compared to the other regions. The Type IV activity was lowest in HP and FC. No differences were seen between control and morphine dependant rats.

Subcellular fractionation revealed Type I PDE to be mostly cytosolic in all brain regions assayed, and Type II to be predominantly membrane-associated, although about evenly distributed in HP. The Type IV PDE subcellular distribution showed the most variation, being mostly particulate in SN and NS, mainly associated with the soluble fraction in LC and DR, and equally distributed in HP and FC.

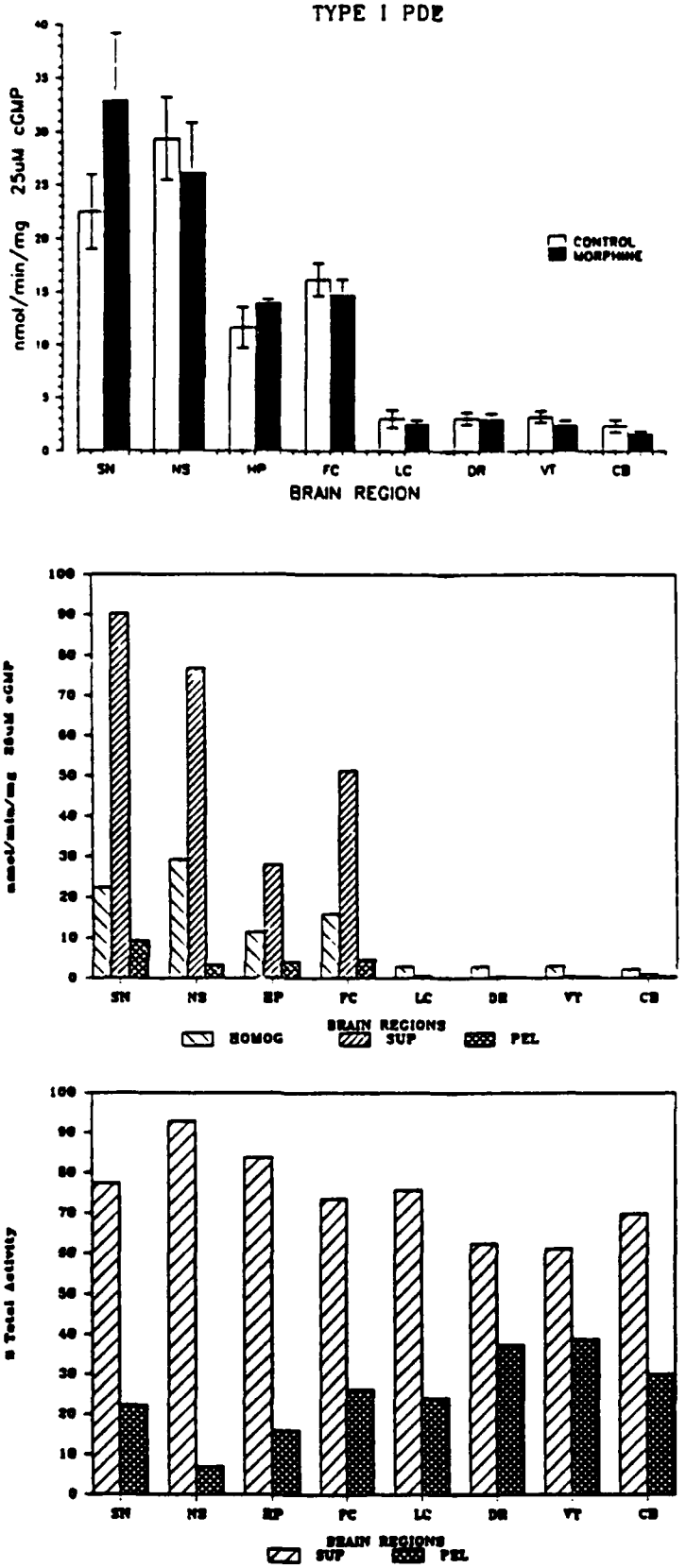

Figure 1.

(a). Basal PDE activity was assayed using 25 uM cGMP as substrate in the presence of 0.2 mM EGTA. The calmodulin - stimulated activity was assayed with 0.4 mM Ca^{++} and 30 nM calmodulin. The stimulated activity (minus basal) represents the Type I PDE activity present as shown here for brain homogenates.

(b). The subcellular distribution of Type I PDE activity was elucidated following 30,000 x g centrifugation of homogenate.

(c). The cytosolic and particulate distribution of Type I PDE expressed as a percent of total activity.

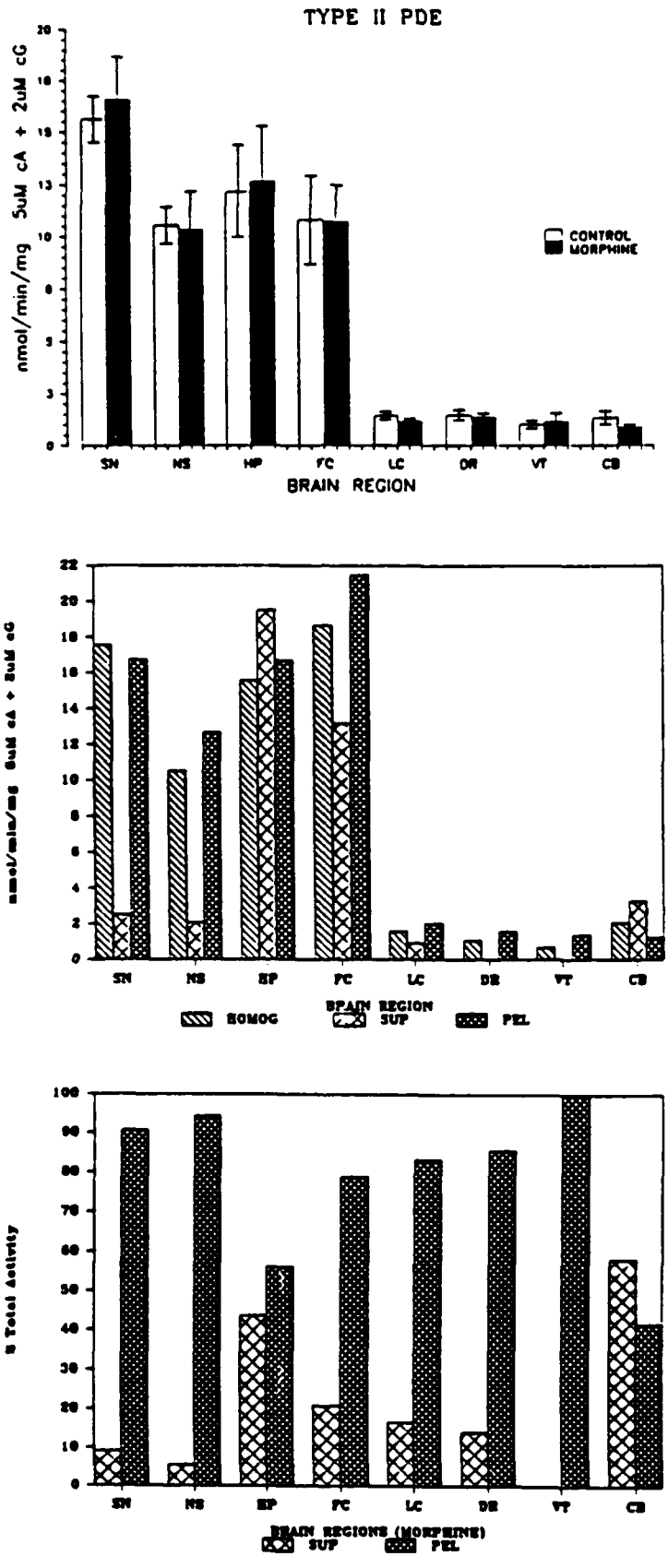

Figure 2.

(a). Type II PDE activity was elucidated in brain homogenates by subtracting 'basal' activity, measured at 5 uM cAMP substrate, from 'stimulated' activity induced by the additional presence of 2 uM cGMP.

(b). The cytosolic and particulate distribution following 30,000 x g centrifugation of homogenates.

(c). The subcellular distribution of Type II PDE activity expressed as a percent of total activity.

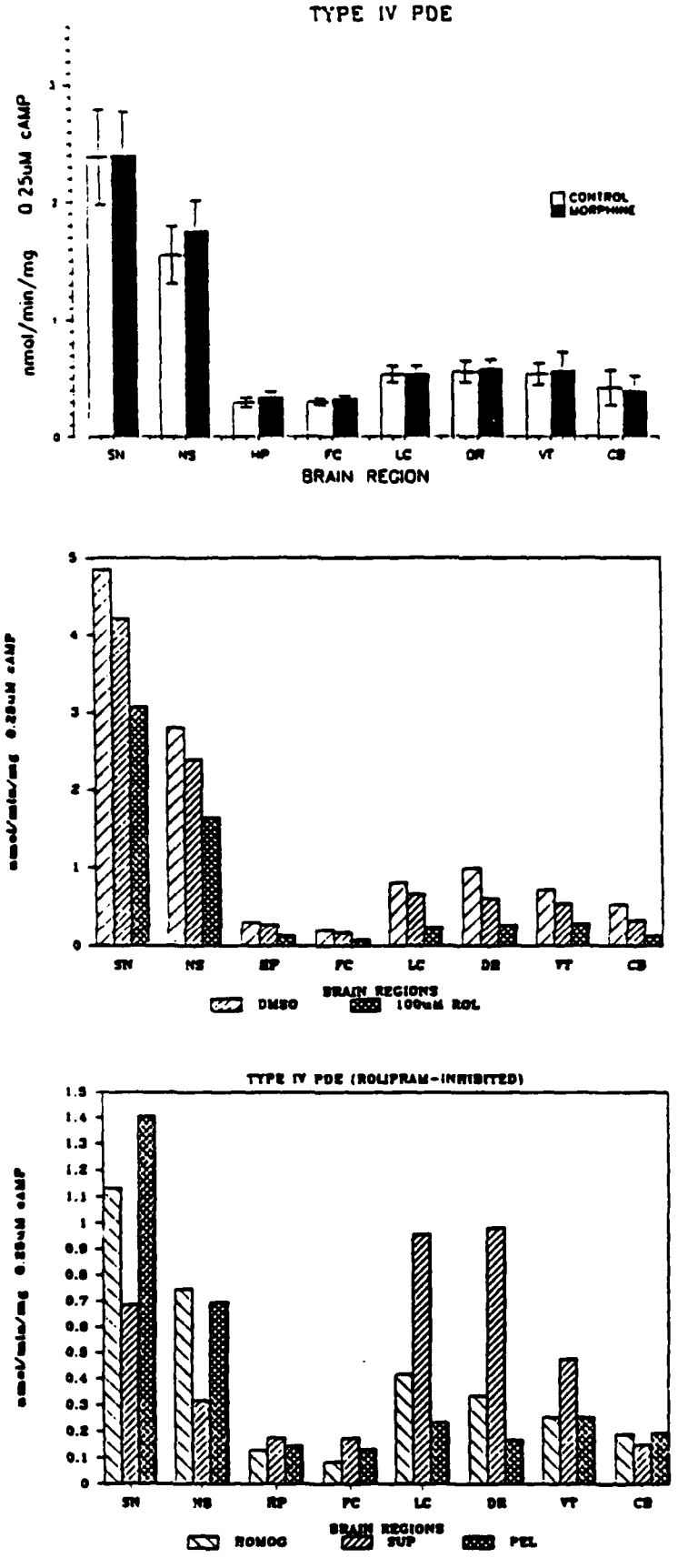

Figure 3.

(a). Type IV PDE activity was assayed at 0.25 uM cAMP substrate.

(b). Type IV activity assayed in the presence and absence of 100uM Rolipram, a specific Type IV inhibitor.

(c). Subcellular distribution of Rolipram-inhibitable PDE activity.

250.19 EFFECTS OF SELECTED PHOSPHODIESTERASE (PDE) INHIBITORS ON CALCIUM-INDEPENDENT PDE ACTIVITY AND ROLIPRAM BINDING SITES OF CEREBRAL CORTEX. L. L. Butcher*, L. A. Lebel* and G. K. Kan. Central Research Division, Pfizer Inc., Groton, CT 06340.

Rolipram, Ro 20-1724 and ICI 63197 are selective PDE inhibitors that are markedly more active on the calcium-independent (cyclic AMP) enzyme (IPDE) than on the calcium-dependent (cyclic GMP) enzyme (DPDE). Recently, membranes, as well as soluble extracts, of various rat brain regions have been reported to contain stereospecific, high affinity binding sites for [³H]rolipram. These binding sites display a linear Scatchard plot and apparently consist of several types characterized by very rapid, moderately fast or very slow dissociation of the radioligand (Schneider et al., Eur. J. Pharmacol. 127: 105, 1986). In the present study, we confirmed the presence of high affinity binding sites for [³H]rolipram in mouse and rat brain preparations and detected these binding sites in several regions of marmoset brain. In addition, we found that the IPDE of rat cerebral cortex (Comp. Biochem. Biophys. Acta 797: 354, 1984) contains high affinity binding sites for [³H]rolipram. Another selective IPDE inhibitor, nitroquazone (TVX 2706; Glaser and Traber, Agents Actions 15: 341, 1984), was found also to inhibit [³H]rolipram binding. High affinity binding to tumor mouse brain preparations and IPDE was found for [³H]nitroquazone, which appears to label the same binding sites as [³H]rolipram. A 2-min association of mouse cortical membranes with either radioligand resulted in binding that was only slowly dissociated by 10 μM rolipram (22 hr).

Since rolipram is a potent inhibitor of IPDE, it was of interest to ascertain if inhibition of IPDE activity correlated with inhibition of binding to rolipram binding sites. For this purpose, we compared the effects of selected PDE inhibitors on hydrolysis of cyclic AMP by rat cortical IPDE and [³H]rolipram (and [³H]nitroquazone) binding to membranes of mouse cerebral cortex. The rank order of inhibitory potency (IC_{50} in nM) on [³H]rolipram or [³H]nitroquazone binding did not parallel the rank order of inhibitory potency on IPDE (IC_{50} in μM) (Table). These results suggest that inhibition of IPDE may be independent of binding to rolipram binding sites.

Compound	IPDE IC_{50} μM	[³H]Rolipram IC_{50} nM	[³H]Nitroquazone IC_{50} nM
Rolipram	0.68	2.8	4.5
(-)Rolipram	0.58	1.8	2.6
GYKI 13238	0.87	15	36
Nitroquazone	1.9	15	18
(+)Rolipram	2.2	5.0	15
Papaverine	4.3	>10000(42%)	>10000(25%)
Proquazone	4.6	7200	10000
Ro 20-1724	6.8	23	50
ICI 63197	6.8	80	100
IBMX	27	700	1500

250.20 REGIONAL VARIATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ISOENZYMES IN DISCRETE BRAIN NUCLEI. R. L. Carroll, IV, W. J. Thompson† and S. J. Strada. University of South Alabama, Department of Pharmacology, College of Medicine, Mobile, AL 36688, and R. S. Duman, E. J. Nestler, and J. F. Tallman, Yale University, Department of Psychiatry, New Haven, CT 06508.

Previous studies have shown considerable variations in cyclic AMP and cyclic GMP phosphodiesterase (PDE) hydrolytic activities among different brain regions (Soc. Neurosci. Abs 384, 1978). To further extend these observations, we analyzed the activities of three distinct types of (PDE) isoenzymes in homogenates of eight brain regions, including five distinct nuclei. These areas included cerebellum (CB), dorsal raphé (DR), hippocampus (HC), locus coeruleus (LC), neocortex (NC), neostriatum (NS), substantia nigra (SN), and ventral tegmentum (VT). Discrete nuclei were excised as 1mm punches from coronal brain sections prepared from 150 g, male, Sprague-Dawley rats. Samples were homogenized with buffer conditions designed to minimize proteolysis (Adv. Cyclic Nucleotide Res. 10: 69-92, 1979).

SN showed the highest Type I cGMP hydrolytic activity (specific activity; 82 nmol/min/mg), measured at 25μM cGMP, and Type IV (high affinity) cAMP PDE activity (8.3 nmol/min/mg), assayed at 0.25μM substrate. These activities were 10-20 fold higher in SN than those measured in other DR or LC. The ratio of cGMP to cAMP hydrolytic activity (G/A ratio) was highest in HP and NC and lowest in SN and NS. Type II (cGMP stimulated) PDE activity, assayed at 3μM cAMP in the absence and presence of 2μM cGMP, showed the greatest cGMP stimulation of cAMP hydrolysis in VT and HP (3.3-3.6 fold), and the least in SN and CB (1.4 fold). Consistent with earlier results, the CB contained the lowest specific activities for each form of PDE. The variations in PDE isoenzyme profiles may have important implications with respect to functional differences in cyclic nucleotide mechanisms among discrete brain nuclei. These studies were supported by a grant from the USPHS (GM 33338) and a contract from the United States Air Force (49620-83-K-0016).

BIOGENIC AMINES: TOXINS

251.1 HISTOCHEMICAL LOCALIZATION OF MPTP OXIDATION BY MAO-B IN SEROTONIN AND HISTAMINE NEURONS IN THE MOUSE BRAIN. S.B. Vincent. Division of Neurological Sciences, Department of Psychiatry, University of British Columbia, Vancouver, B.C., V6T 1W5, Canada.

Conversion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to 1-methyl-4-phenylpyridinium (MPP^+) appears to be a necessary step for the neurotoxic actions of this drug on the nigrostriatal dopamine system. This conversion is mediated by monoamine oxidase (MAO) of the B type. In the present study, MPTP has been used as a substrate for the histochemical localization of MAO activity in the brain of CS7 black mice. The localization of MPTP oxidation by MAO in the brain was compared with the distribution of various monoaminergic neurons determined using immunohistochemistry.

Adult male CS7 black mice were anesthetized and perfused with buffered aldehyde fixative. MAO activity was demonstrated on 50 μm thick vibratome sections by incubating the sections in 50 mM Tris-Cl buffer (pH 8.5) containing 0.9% MPTP hydrochloride, 0.1% horseradish peroxidase, 0.005% diaminobenzidine, 0.6% nickel ammonium sulfate and 0.9% sodium azide. Sections incubated without MPTP showed no positive reaction. For the immunohistochemical localization of catecholamine neurons sections were incubated with antisera against tyrosine hydroxylase, dopamine-β-hydroxylase and phenylethanolamine-N-methyltransferase. Serotonin and histamine neurons were localized with antibodies to serotonin and histidine decarboxylase.

The distributions of monoamine cell groups observed in the mouse brain were similar to those found in the rat. Tyrosine hydroxylase immunohistochemistry demonstrated that the major dopaminergic cell group in the mouse was in the substantia nigra pars compacta and adjacent ventral tegmental area. These dopamine neurons did not display MAO activity when MPTP was employed as a substrate. Instead, MAO activity capable of oxidizing MPTP was found in other discrete groups of neurons. These included the serotonin and noradrenaline neurons of the brainstem, and the histamine neurons of the caudal hypothalamus. Preincubation of sections with the MAO-A inhibitor clorgyline blocked the MAO staining in noradrenergic neurons, but not in the serotonin or histamine neurons. The activity in these cell groups was inhibited by the MAO-B inhibitor deprenyl.

These results indicate that MPTP can be converted to the Parkinsonism-inducing toxin MPP^+ by MAO-B in serotonin and histamine neurons which innervate the striatum and substantia nigra.

Supported by the British Columbia Health Care Research Foundation.

251.2 MPTP EFFECTS ARE REGIONALLY SPECIFIC IN MICE - A NEUROCHEMICAL STUDY. M. Gupta, S.Y. Felten, and D.L. Felten. Department of Neurobiology and Anatomy, University of Rochester School of Medicine, Rochester, NY 14642.

MPTP causes degeneration of the nigrostriatal dopamine system in humans, non-human primates, and rodents. Although degeneration of the nigrostriatal dopaminergic neurons is the most prominent abnormality in human Parkinsonism, additional monoamine cell groups also are known to be affected by this disease. We previously have shown that MPTP treatment in young adult mice also leads to decreased dopamine levels in the nucleus accumbens and olfactory tubercle(dopamine mesolimbic projection sites from neurons of the ventral tegmental area) in a dose-dependent manner, in addition to decreased dopamine levels in the caudate-putamen (Gupta et.al., in: MPTP-A neurotoxin producing a Parkinsonian syndrome, eds. Markey et.al., 1985). In the present study, we investigated whether MPTP treatment in mice produces changes in other monoamines in addition to its already established changes in dopamine levels. Young adult male Swiss-Webster mice were injected intraperitoneally with 3, 30, or 60mg MPTP/kg body weight. Control animals received vehicle injections. Treated and control animals were sacrificed by decapitation 21 days following the last injection. Brains were removed quickly and samples from various regions of the brain, both nuclei and terminal sites, were microdissected, placed in 100mM perchloric acid, frozen and stored in liquid nitrogen. Levels of monoamines were determined using high performance liquid chromatography with electrochemical detection. MPTP treatment increases norepinephrine (NE) levels in the ventral tegmental area and decreases NE levels in substantia nigra, whereas no changes were seen in locus coeruleus, mediobasal hypothalamus, dorsal raphe, medullary raphe, and nucleus tractus solitarius. Furthermore, serotonin levels appeared to be decreased in substantia nigra in a dose-dependent manner, but remained unaltered in dorsal raphe, medullary raphe, mediobasal hypothalamus, and nucleus tractus solitarius. We conclude that MPTP affects only specific monoaminergic regions of the brain while leaving other regions unaltered.

Supported by USPHS grants RO1 AG06060, RO3 MH41435, and R23 NS24291.

Project # 2

REGIONAL AND SUBCELLULAR DISTRIBUTION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE (PDE) ISOZYMES IN RAT BRAIN.

Methods.

Male Sprague Dawley rats (200-350g) were sacrificed by decapitation and brains rapidly removed and placed in cold Krebs-Ringer buffer, (pH 7.4) containing glucose. Larger brain areas, e.g. frontal cortex, neostriatum, substantia nigra, hippocampus, ventral tegmentum, and cerebellum were grossly dissected using a standard rat brain atlas. The locus coeruleus and dorsal raphe were isolated by taking 15 gauge punches from 0.5 to 0.75mm thick coronal cross-sections of brain. For regional distribution of PDE activities, brain tissue was homogenized with buffer conditions designed to minimize proteolysis (Adv. Cyclic Nucleotide Res. 10: 69-92, 1979) and aliquots assayed for the three PDE isozymes. For subcellular distribution studies, homogenates of the various brain regions were centrifuged at 30,000 x g, 15 min. with the resulting supernatants and pellets assayed for the three PDE isozymes. Cortical membranes were further fractionated by the method of Dodd et al. (Brain Res. 226:107-118,1981).

Results

Regional and subcellular distribution of PDE isozymes [Type I (Ca^{2+}/calmodulin-sensitive), Type II (cGMP-activatable) and Type IV (high affinity cAMP specific)] was examined in eight rat brain regions. Substantia nigra (SN), neostriatum (NS), frontal cortex (FC), and hippocampus (HP) contained highest specific activity (S.A.) Type I and Type II PDE, while SN and NS contained highest S.A. Type IV PDE. Subcellular fractionation revealed Type I PDE is cytosolic in all brain regions, Type II PDE is predominately membrane-associated and Type IV PDE is distributed equally between compartments. Further fractionation of cortical membranes showed that Types II and IV PDE reside in synaptosomes. Combined studies using immunoprecipation

and pharmacological selectivity indicate that the Type II PDE is the predominate form in synaptosomes. The results support the notion that different PDE isozymes exert preferential hydrolytic roles in various brain regions and subcellular compartments.

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REGIONAL AND SUBCELLULAR DISTRIBUTION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE (PDE) ISOZYMES IN RAT BRAIN.

M.E Whalin*, W.J. Thompson, R.L. Garrett, Jr.* and S.J. Strada
Dept. of Pharmacology, Univ. of South Alabama College of Medicine, Mobile, AL 36688.

The regional and subcellular distribution of PD isozymes [Type I (Ca^{2+}/calmodulin-sensitive), Type II (cGMP sensitive) and Type IV (high affinity cAMP specific)] wa examined in eight rat brain regions. Substantia nigr (SN), neostriatum (NS), frontal cortex (FC), and hippo campus (HP) contained highest specific activity Type I an Type II PDE, while SN and NS contained highest specifi activity Type IV PDE. Subcellular fractionation reveale Type I PDE is cytosolic in all brain regions, Type II PD is predominately membrane-associated and Type IV PDE i distributed equally between compartments. Further frac tionation of cortical membranes showed that Types II and I PDE reside in synaptosomes. Combined studies using immuno precipation and pharmacological selectivity indicate tha the Type II PDE is the predominate form in synaptosomes The results support the notion that different PDE isozyme exert preferential hydrolytic roles in various brai regions and subcellular compartments. These studies wer supported by grants from the USPHS (GM33538) and the USA (49620-85-K-0014).

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250.15 SPATIAL AND TEMPORAL EXPRESSION OF mRNA ENCODING THE α SUBUNIT OF G_o: MAPPING IN RAT BRAIN BY IN SITU HYBRIDIZATION. R.R. Reed*, R.L. Lazzarini, D.T. Jones*, C. Frenzen*, P.P. Worley, and S.H. Snyder, (SPON: K. Brass) Dept. of Molecular Biology and Genetics, Howard Hughes Medical Institute, and Dept. of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

The G-proteins represent a family of proteins that bind guanine nucleotides and reversibly participate in many signal transduction processes. Members of this G-protein family are highly conserved at the protein and nucleotide level. To distinguish in our studies among various members of this family, we generated specific oligonucleotide probes to unique 3' untranslated regions of each G-protein cDNA. These probes were used to detect mRNA abundance and distribution in rat brain. The oligonucleotide probes were labeled using a novel technique involving the synthesis of a 38mer oligonucleotide containing 46 nucleotides of target-specific sequence followed by an additional 12 nucleotides (CAR-common annealing region). A second oligonucleotide (22mer), consisting of T_{10} followed by the sequence complementary to the CAR region, was annealed to the 38mer oligo and extended with α-^{35}S-dATP and DNA polymerase. These probes of defined specific activity and uniform length were then used for in situ hybridization studies.

G_o, the stimulatory G-protein, is associated with the receptor-mediated activation of adenylyl cyclase, an enzyme abundant in neural tissues and essential for many types of neurotransmission. Mapping of mRNA encoding the α subunit of G_o reveals abundant hybridization and heterogeneous distribution throughout the brain. Control studies confirm the hybridization specificity. G_o mRNA is prevalent throughout the brain, being particularly evident in large neurons such as pyramidal cells of the piriform cortex and hippocampus, and neurons of motor nuclei and reticular formation. Interestingly, the general pattern of hybridization is quite similar to the immunocytochemical distribution of adenosine (K. Brass, et al, J. Neurosci., 6:1952-1961, 1986). Localization of G_o message does not parallel [^{3}H]forskolin binding (a marker for G_s-coupled adenylyl cyclase) in a fashion that might be expected. Notably, cells of the caudate nucleus, whose neurons contain the highest levels of adenylyl cyclase in the brain, demonstrate the lowest abundance of G_o mRNA. This mismatch may suggest that adenylyl cyclase does not exclusively couple through G_o. Alternatively, G_o in other areas of the brain may effect transduction through other second messenger enzymes. Presently, we are examining the modulation of G_o message levels after intracranial injection of the bacterial toxins cholera and pertussis, known to covalently modify G_o subunits. Additionally, the ontogeny of G_o mRNA in embryonic and neonatal brain is being assessed.

250.16 BINDING OF GUANINE NUCLEOTIDE ANALOGUES TO ADENYLATE CYCLASE REGULATORY PROTEINS IN NEURAL CELL MEMBRANES. J.M. Gordon, M.H. Harrow* and M.H. Rasenick, The Chicago Med Schl, N. Chicago, IL 60064 and Dept Physiol and Biophys, U. of Illinois College of Med, Chicago, IL 60680.

Neural cell membranes, when exposed to hydrolysis-resistant analogues of GTP, show a dose-dependent stimulation or inhibition (depending upon assay conditions) of adenylate cyclase (AC). The activation or inhibition of AC persist subsequent to washing of membranes and is independent of neurotransmitter(s). Although the binding of GTP analogues to detergent-solubilized and purified guanine nucleotide binding regulatory proteins (GR) has been studied, the kinetics of guanine nucleotide binding to individual GR in membranes has not been characterized.

Rat cerebral cortex membranes were incubated with either varying concentrations of the hydrolysis-resistant, photoaffinity GTP analog, azidoanilido GTP (^{32}P-AAGTP) or with a constant amount of ^{32}P-AAGTP and varying concentrations of cold GTP analogues. Membranes were incubated for 3 min at 21°C, centrifuged, resuspended in fresh buffer and exposed to UV irradiation for 3 min. Following irradiation the suspension was centrifuged. The resulting pellet was dissolved in sample buffer, subjected to SDS-PAGE and radioautography. Radioactive bands corresponding to AC stimulatory GR (52 and 42 KDa bands; GR_{52} and GR_s), inhibitory GR (40 KDa doublet composed of GR_i and GR_o) and a novel neural GR (32 KDa band; GR_{32}) were excised from the dried gels and the amount of radioactivity quantitated.

Isotherm analysis revealed that the $GR_{i/o}$ band had the highest affinity for ^{32}P-AAGTP, K_D= 1.12±0.38 nM (mean ± SE), followed by GR_{32} (3.39±0.39), GR_s (4.87±0.66) and GR_{52} (5.70±1.23). Separation of the $GR_{i/o}$ doublet into its components (i.e. GR_i and GR_o) indicated that these two GR were not significantly different in their binding affinity for nucleotide. Analysis of competition assays indicated that all of the guanine nucleotides studied were similar to AAGTP as they all displayed the highest affinity for the $GR_{i/o}$ band and the lowest affinity for the GR_{52}. Of the GTP analogues studied GTPγS displayed the highest affinity for all GR followed by GDPβSGTPγSAAGTP>GTP. Cppidp and AAGTP displayed a significantly higher affinity for $GR_{i/o}$ than GR_s; whereas GTPγS GDPβS and GTP (which is rapidly hydrolyzed to GDP) displayed nearly equal affinity for both proteins. Whether these observations are a result of assay conditions or innate affinities is not known at this time. These data indicate that components of synaptic membranes may alter apparent affinity of GR for nucleotide and that the activation/inhibition of AC is a dynamic process with multiple sites of regulation.

250.17 ISOLATION AND CHARACTERIZATION OF THE INOSITOL 1,4,5 TRISPHOSPHATE (IP_3) BINDING SITE. S. Supattapone, P. Worley, J. Baraban and S.H. Snyder. Department of Neuroscience, Johns Hopkins Univ. Sch. of Med., Baltimore, MD 21205

IP_3 appears to be the second messenger responsible for mobilizing calcium from internal stores (Berridge, M.J., and Irvine, R.F. Nature, 312:315, 1984). Therefore, it would be of interest to characterize the binding site for IP_3 in the cell. Previous reports (Worley, P.L., et al., Nature, 325:159, 1987) have demonstrated that the rat cerebellum is a very abundant source of a high affinity IP_3 binding site.

In this study, we have solubilized and purified the IP_3 binding site to apparent homogeneity from rat cerebellum. The purified receptor is globular and has a Stokes' radius of 18 nm. Therefore, its molecular weight is on the order of one million daltons. While IP_3 binding is reversibly inhibited by 300 nM calcium in crude homogenates and solubilized membranes, the purified binding site is not inhibited by calcium concentrations up to 1.5 mM. Inhibition by calcium could be reconstituted by addition of crude solubilized cerebellar membranes, but not by the cytosolic fraction of cerebellum.

250.18 IDENTIFICATION AND PURIFICATION OF BRAIN TYPE II PHOSPHODIESTERASE: A DISTINCT cGMP RECEPTOR PROTEIN IN MAMMALIAN BRAIN MEMBRANES. M.E. Whalin*, Mark Thaeppon*, and S.A. Strada (SPON: H.E. Langenecker). University of South Alabama, Dept. of Pharmacology, College of Medicine, Mobile, AL 36688.

Type II (cGMP stimulated) cyclic nucleotide phosphodiesterase(PDE) as purified from heart, liver, and adrenal tissues shows a preference for cGMP as substrate and displays enhanced cAMP hydrolysis by low, physiological concentrations of cGMP. Our studies of brain Type II PDE indicate it to be the majority of the hydrolytic activity found in membrane fractions. It is not released by either hypotonic or high ionic strength buffers. Detergent solubilization of the Type II PDE does not preserve its regulation by cGMP. However, if released by limited proteolysis using TPCK-trypsin, full cGMP regulation is retained. The solubilized enzyme was purified to apparent homogeneity, utilizing DEAE-cellulose anion exchange, cGMP epoxy-sepharose 4B, and hydroxylapatite chromatography. A 3000 fold increase in specific activity was observed. Its Mr is 240 kD by gel filtration. The subunit Mr of the enzyme determined by SDS-PAGE analysis (7.5%) shows a major protein band at 103-105 kD. Maximum velocities are 157 U/mg and 159 U/mg for cAMP and cGMP respectively. $S_0.5$ are 28 uM for cAMP and 16 uM for cGMP. The Kact for cGMP stimulation of cAMP hydrolysis at 5 uM substrate is 0.33 uM and maximum stimulation (3 fold) is achieved at 2 uM cGMP. The purified enzyme is phosphorylated by the catalytic subunit of cAMP dependent protein kinase and retains the same subunit Mr. Phosphorylation does not appear to affect cAMP hydrolysis at 5 uM substrate in the absence or presence of 2 uM cGMP, but does reduce cGMP hydrolysis measured at 40 uM substrate by 30%. Monoclonal antibodies produced against purified Type II PDE immunoprecipitate enzyme activity (>90%) with the immunoprecipitate retaining full regulation by cGMP. Immunocytochemical studies are being pursued to define the regional distribution and localization of the Type II PDE observed by activity analysis in related studies (Garrett et al., this volume). This enzyme may constitute a major cGMP receptor and may serve an important regulatory role in controlling the level of cyclic nucleotides during neuronal function. These studies were supported by USPHS (GM 33538) and a contract from the U.S. Air Force (49620-85-K-0014).

Project # 3

DIFFERENTIAL SENSITIVITY TO CYCLIC NUCLEOTIDE PHOSPHODIESTERASE INHIBITORS IN RAT BRAIN CORTICAL SLICES.

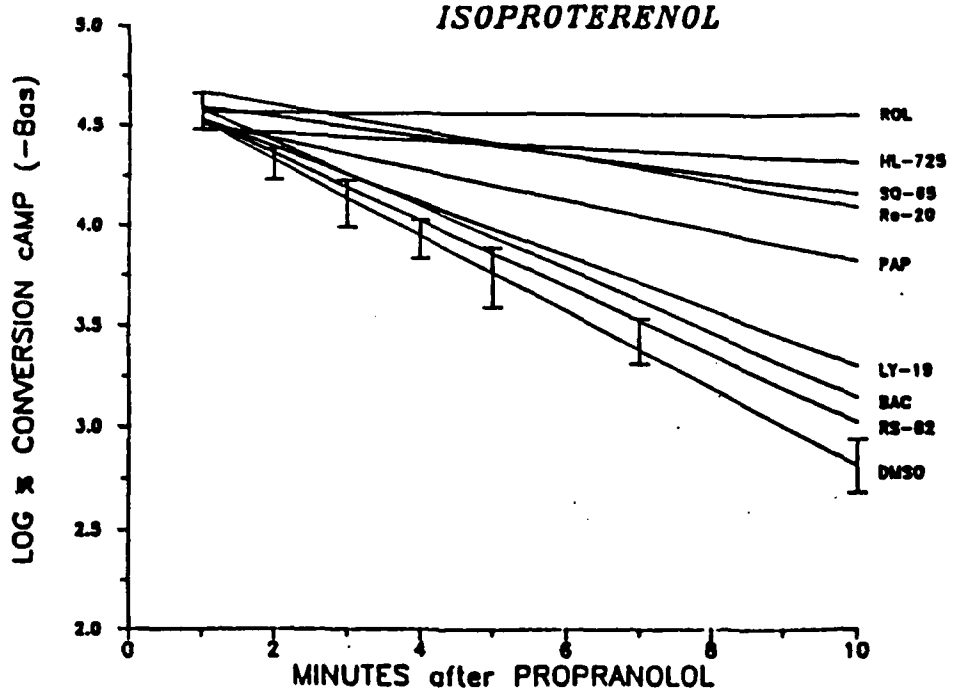
These studies are examining the effects of various cyclic nucleotide phosphodiesterase inhibitors on the turnover rate of cyclic AMP in brain slices after stimulation of different receptor systems.

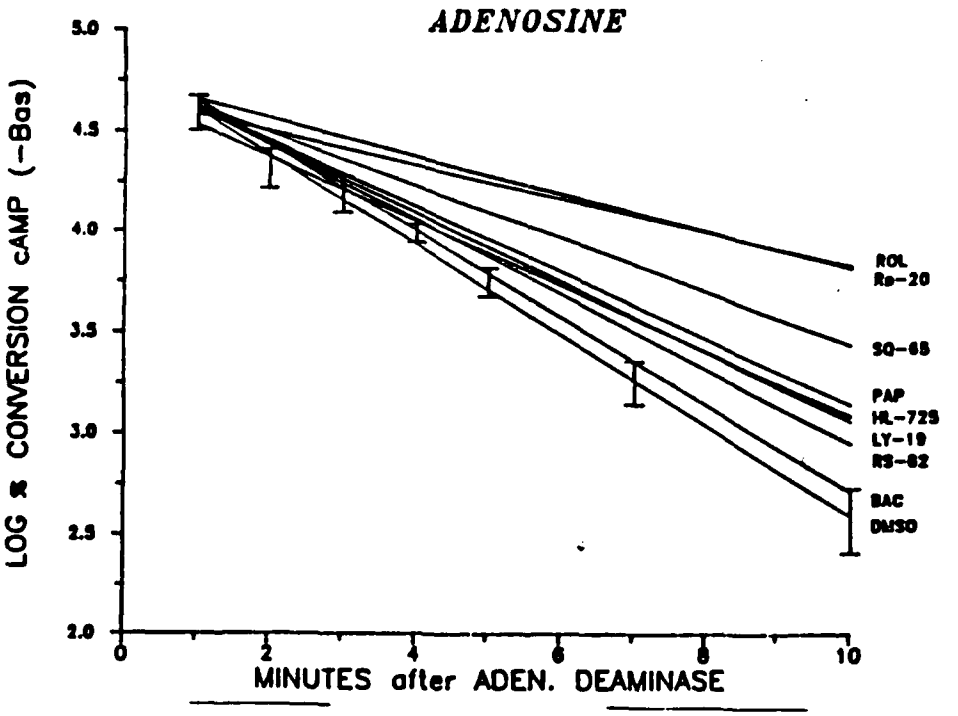
PROCEDURE:

Male, Sprague-Dawley rats (200-225g) were killed by decapitation, the brains rapidly removed into ice-cold buffer (10mM Hepes, 154mM NaCl, pH 7.4), and the cortex excised. Slices were prepared using a McIlwain chopper (0.22mm setting), then transferred to oxygenated Krebs-Ringer buffer containing glucose maintained at 37°C for a 15 min. equilibration period, during which the buffer was changed three times. The slices were then incubated for one hr. with [^{3}H]-adenine, then washed and incubated with maximally effective concentrations of 20uM isoproterenol (ISO) or 50uM adenosine for 12.5 min. After settling, 50ul of slices were rapidly transferred into buffer containing 200uM propranolol or 0.55 units adenosine deaminase, and 80uM drug or equivalent volume of DMSO. This incubation period was stopped by the addition of an equal volume of 10% TCA at varying time points ranging from 1-20 min. Following homogenization, the samples were centrifuged at 20,000xg for 10 min., the cAMP extracted from the supernatant and isolated by the Dowex AG50/Alumina double column method, and the % conversion and decay constant determined as described by Barber et al. (Mol. Pharm. 32:753, 1987). (The decay constant is the negative slope of the straight line obtained by plotting the natural log of the response (% conversion - basal) versus time.)

RESULTS:

Rolipram was the best inhibitor of cAMP decline in both receptor systems, showing 60% inhibition following adenosine stimulation of cAMP levels, and >90% inhibition in the ISO system. Other PDE-inhibitors tested (eg. HL-725, SQ-65442) also showed greater efficacy against decline in cAMP levels following ISO stimulation than adenosine stimulation, while some (eg. Ro 20-1724) did not appear to discriminate between the two systems. Some drugs (e.g. RS-82856, LY-195115) were poor inhibitors in both systems. This variation in inhibitor susceptability may indicate the involvement of separate PDE isoenzymes linked to different agonist receptor systems.





The effect of various drugs (80 uM) on the decay rate of cAMP in rat cortical slices was measured according to the procedure described in "methods". Baclofen does not appear to significantly affect the cAMP decay rate compared to known PDE inhibitors.

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DIFFERENTIAL SENSITIVITY TO CYCLIC NUCLEOTIDE PHOSPH DIESTERASE INHIBITORS IN RAT BRAIN CORTICAL SLICES. R. Garrett, Jr.*, W.J. Thompson, M.E. Whalin* and S.J. Stra Dept. of Pharmacology, Univ. of South Alabama College Medicine, Mobile, AL 36688.

The effects of selective cyclic nucleotide phosphodi terase (PDE) inhibitors on the rate of cyclic AMP (cA turnover were examined in male, rat cortical slices. The conversion of [^{3}H]-labeled adenine nucleotide pools i cAMP was determined after stimulation with maxima effective concentrations of isoproterenol (ISO) adenosine (AD). The decay constant was measured accord to Barber et al. (Mol. Pharm. 32:753, 1987) using addition of either 200 µM propranolol or 0.55 units of deaminase to terminate the agonist response. Rolipram the best inhibitor of cAMP decline in both receptor syste However, the drug showed only 60% inhibition following stimulation of cAMP levels, while >90% inhibition in ISO stimulated system. Other PDE-inhibitors tested (HL-725, SQ-65442) also showed greater efficacy against decline in cAMP levels following ISO than after AD. S drugs (eg. RS-82856,LY-195115) were poor inhibitors also did not discriminate between the two systems. T variation in inhibitor sensitivity may indicate the invol ment of separate PDE isozymes linked to different agon receptor systems. These studies were supported by gra from the USPHS (GM 33538) and the USAF (49620-85-K-0014).

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Rapid Communication

γ-Aminobutyric Acid$_B$ Receptor Activation Modifies Agonist Binding to β-Adrenergic Receptors in Rat Brain Cerebral Cortex

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Abstract: The interaction of isoproterenol with β-adrenergic receptor (βAR) binding sites was measured in membranes prepared from rat brain cerebral cortical slices previously incubated in the presence or absence of γ-aminobutyric acid (GABA) receptor agonists. Both GABA and baclofen, but not isoguvacine, altered βAR agonist binding by increasing the affinity of both the low- and high-affinity binding sites and by increasing the proportion of low-affinity receptors. The response to baclofen was stereoselective, and the effect of GABA was not inhibited by bicuculline. The results suggest that GABA$_B$, but not GABA$_A$, receptor activation modifies the coupling between βAR and stimulatory guanine nucleotide-binding protein, which may in part explain the ability of baclofen to augment isoproterenol-stimulated cyclic AMP accumulation in brain slices. **Key Words:** γ-Aminobutyric acid—Baclofen—β-Adrenergic receptor—Isoproterenol—GABA$_B$ receptors—Brain membranes—Cyclic AMP. **Scherer R. W. et al.** γ-Aminobutyric acid$_B$ receptor activation modifies agonist binding to β-adrenergic receptors in rat brain cerebral cortex. *J. Neurochem.* **53**, 989–991 (1989).

There are at least two receptor subtypes for γ-aminobutyric acid (GABA): GABA$_A$ and GABA$_B$. The GABA$_A$ receptors are associated with Cl^- flux (Enna and Gallagher, 1983), whereas GABA$_B$ receptor activation alters K^+ and Ca^{2+} channels (Gahwiler and Brown, 1985; Feltz et al., 1987) and modifies second messenger production. With regard to second messengers, baclofen, a selective GABA$_B$ receptor agonist (Hill and Bowery, 1981), augments neurotransmitter-stimulated cyclic AMP accumulation in brain slices while having no direct effect on second messenger formation (Hill, 1985; Karbon and Enna, 1985; Watling and Bristow, 1986). The findings that baclofen reduces adenylate cyclase activity in brain homogenates and diminishes forskolin-stimulated cyclic AMP production in rat brain slices (Wojcik and Neff, 1984; Karbon and Enna, 1985) suggest the existence of pharmacologically distinct subsets of GABA$_B$ receptors (Scherer et al., 1988).

Although the GABA$_B$ receptor-mediated inhibition of adenylate cyclase in homogenates appears to be mediated by a guanine nucleotide binding protein (G_i or G_o) (Xu and Wojcik, 1986), the cellular components associated with the cyclic AMP-augmenting response to baclofen are unknown (Karbon and Enna, 1985). In addition to increasing the amount of neurotransmitter-stimulated cyclic AMP, baclofen increases the potency of neurotransmitters to stimulate accumulation of this second messenger in brain (Karbon et al., 1984; Karbon and Enna, 1985). Because receptor-mediated cyclic AMP production requires the coordinated action of several factors—including the receptor, the stimulatory and inhibitory guanine nucleotide-binding proteins (G_s and G_i), and the catalytic unit of adenylate cyclase (Allende, 1988)—it is conceivable that baclofen modifies this system by influencing one or more of these components. Given the observation that baclofen may influence the potency of receptor agonists to stimulate cyclic AMP accumulation, the present study was undertaken to examine the effect of GABA$_B$ agonists on β-adrenergic receptor (βAR) agonist binding in rat brain cerebral cortical membranes.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Charles River), weighing 200–300 g, were decapitated, and the brains were removed and placed into ice-cold HEPES-buffered saline (154 m*M* NaCl and 10 m*M* HEPES, pH 7.4). The frontal cortex was dissected, blotted dry, and weighed. Portions of tissue (40–100 mg wet weight) were minced with a McIlwain tissue chopper (0.26 × 0.26 mm) and immediately placed into vials containing 5 ml of Krebs-Ringer-bicarbonate buffer (37°C), aerated with 95% O_2/5% CO_2, of the following composition (in m*M*): NaCl, 118; KCl, 5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.2; $NaHCO_3$, 25; and D-glucose, 11.1. Either vehicle (50 μl)

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Abbreviations used: βAR, β-adrenergic receptor; GABA, γ-aminobutyric acid; G_i and G_s, inhibitory and stimulatory guanine nucleotide-binding proteins, respectively.

or test substance was added to the slice-containing solution and incubated for 10 min. The aqueous portion was aspirated, and the slices were homogenized (Brinkmann Polytron, setting of 5.5 for 5–10 s) in 5 ml of HEPES buffer (1 m*M* Na_2EDTA and 2m*M* HEPES, pH 7.5) containing the same concentration of test agent. The homogenate was centrifuged (48,000 *g*, 10 min), and the pellet was resuspended and washed three additional times in a similar manner before suspension (6.5 mg of tissue/ml) in Tris-buffered saline (154 m*M* NaCl, 2.5 m*M* $MgCl_2$, and 20 m*M* Tris, pH 7.4) (O'Donnell et al., 1984).

Saturation binding of [^{125}I]iodopindolol was performed with radioligand concentrations ranging from 10 to 1,000 p*M* (O'Donnell et al., 1984). Specific binding was defined as that portion of the total binding displaced by 100 μM isoproterenol. The samples were incubated for 30 min at 37°C, and the reaction was terminated by addition of 5 ml of ice-cold Tris buffer and filtration over GF/B filters in a Brandel cell harvester. The filters were washed four times each with 4 ml of buffer, after which radioactivity was quantified using a gamma counter.

Competition experiments were conducted with 100 p*M* [^{125}I]iodopindolol and isoproterenol concentrations ranging from 0.1 to 10,000 n*M*. Least squares analysis was performed using Lundon 2 (Lundon Software, Chagrin Falls, OH, U.S.A.). Two binding sites were deemed more likely than one if the *F* statistic for the former was associated with a *p* value of ≤0.05.

To assess accurately the effect of GABA agonists on βAR agonist binding, the data from drug-treated tissue were compared only with control data obtained in parallel experiments. Therefore, on a given day, all tissue slices originated from the same pool of tissue. Statistical analysis was performed using a two-tailed Student's *t* test. Differences were considered significantly different for a *p* value of ≤0.05.

Protein concentrations were determined using reagent kits from Bio-Rad (Richmond, CA, U.S.A.).

Baclofen (D,L-, D-, and L-) was kindly supplied by Ciba-Geigy (Summit, NJ, U.S.A.). [^{125}I]Iodopindolol was purchased from New England Nuclear (Boston, MA, U.S.A.), and isoproterenol, bicuculline methiodide, and GABA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Isoguvacine was purchased from Research Biochemicals, Inc. (Natick, MA, U.S.A.).

RESULTS

In control rat brain cortical membranes, the K_D for [^{125}I]iodopindolol binding was 86 p*M*, and the receptor density was 150 fmol/mg of protein. Competition experiments with isoproterenol yielded shallow displacement curves. When examined using least squares analysis, the data were best described by a two-site model. Under these assay conditions, the equilibrium dissociation constants for the low- (K_L) and high- (K_H) affinity states for isoproterenol ranged from 148 to 234 and 21 to 40 n*M*, respectively (Table 1). Exposure of brain slices to D,L-baclofen (100 μM) increased the affinity of both the high- and low-affinity states for isoproterenol. The percentage of low-affinity agonist binding sites increased nearly 50% following exposure to the $GABA_B$ agonist, whereas the percentage of high-affinity sites was significantly reduced (Table 1). Exposure of cortical slices to baclofen did not affect [^{125}I]iodopindolol binding affinity (K_D = 84 p*M*), nor did the $GABA_B$ agonist influence βAR binding when added directly to membranes prepared from slices exposed to vehicle alone (data not shown).

The effect of baclofen was stereoselective, with L-baclofen (10 μM) and the racemate altering β-adrenergic agonist binding, whereas D-baclofen (10 μM) was ineffective. Exposure to 1 μM L-baclofen shifted the proportion of low- and high-affinity agonist binding sites, without causing a significant change in receptor affinity.

When cortical slices were incubated in the presence of 25 μM GABA, the competition curve for isoproterenol was best described as a single site with intermediate affinity (Table 2). Bicuculline (50 μM), a $GABA_A$ receptor antagonist, did not attenuate the response to GABA, and isoguvacine (20 μM), a selective $GABA_A$ receptor agonist, had no effect on βAR binding (Table 2).

DISCUSSION

The results of this study indicate that exposure of rat brain cerebral cortical slices to $GABA_B$, but not $GABA_A$, receptor agonists alters βAR agonist binding. Agonist attachment to both low- and high-affinity sites is enhanced by $GABA_B$ agonists, as is the proportion of the low-affinity receptors.

Under control conditions, the displacement curve repre-

TABLE 1. *Stereoselective effect of baclofen preincubation on isoproterenol displacement of [^{125}I]iodopindolol binding to rat brain cerebral cortical membranes*

Condition (n)	K_H (n*M*)	K_L (n*M*)	% K_H	% K_L
Control (7)	26 ± 3	222 ± 31	54.4 ± 6	45.6 ± 6
100 μM D,L-baclofen (8)	12 ± 2[a]	134 ± 12[a]	32.2 ± 6[a]	67.8 ± 6[a]
Control (5)	40 ± 5	234 ± 21	57.7 ± 9	42.3 ± 9
10 μM L-baclofen (5)	12 ± 2[a]	135 ± 12[a]	25.9 ± 5[a]	74.1 ± 5[a]
10 μM D-baclofen (4)	45 ± 5	237 ± 54	52.2 ± 6	47.8 ± 6
Control (4)	21 ± 2	148 ± 16	34.2 ± 1	65.8 ± 1
1 μM L-baclofen (5)	16 ± 4	119 ± 12	22.8 ± 3[a]	77.2 ± 3[a]

Competition by isoproterenol against [^{125}I]iodopindolol binding was measured in membranes prepared from rat brain cerebral cortical slices incubated for 10 min in the absence (control) or presence of baclofen. Data are mean ± SEM values from four to eight separate experiments (shown in parentheses). The data from drug-treated groups were compared only with control data obtained from experiments done in parallel. K_H and K_L are the dissociation constants for isoproterenol at the high- and low-affinity sites, respectively, whereas % K_H and % K_L are the percentages of isoproterenol bound to the high- and low-affinity sites, respectively.

[a] Significantly different from the corresponding control ($p < 0.05$ by Student's two-tailed *t* test).

TABLE 2. *Effect of GABAergic compounds on isoproterenol displacement of [^{125}I]iodopindolol binding to rat brain cerebral cortical membranes*

Condition (n)	K_H (nM)	K_L (nM)	% K_H	% K_L
Control (5)	17 ± 2	195 ± 25	33.9 ± 5	66.1 ± 5
25 μM GABA (4)	[a]	92 ± 12[b]	[a]	95.6 ± 4[b]
20 μM isoguvacine (5)	18 ± 4	183 ± 9	33.1 ± 5	66.9 ± 5
50 μM bicuculline (5)	20 ± 4	199 ± 13	41.5 ± 5	58.5 ± 5
GABA + bicuculline (4)	[a]	119 ± 24[b]	[a]	92.8 ± 6[b]

Competition by isoproterenol against [^{125}I]iodopindolol was measured in membranes prepared from rat brain cerebral cortical slices incubated for 10 min in the absence (control) or presence of various agents. Data are mean ± SEM values from four or five separate experiments (shown in parentheses). In a given experiment, all of the drug-treated groups were examined simultaneously with controls. K_H and K_L are the dissociation constants for isoproterenol at the high- and low-affinity sites, respectively, whereas % K_H and % K_L are the percentages of isoproterenol bound to the low- and high-affinity sites, respectively.

[a] In three of four experiments, the curve was best fitted by a single-site model.

[b] Significantly different from the control ($p < 0.05$ by Student's two-tailed t test).

senting the interaction of isoproterenol with [^{125}I]iodopindolol binding was quite shallow, a result indicating multiple sites (O'Donnell et al., 1984). Previous reports (DeLean et al., 1980) suggested that βAR agonists bind with low affinity to an uncoupled receptor, whereas high-affinity binding reflects the formation of a ternary complex comprising agonist, βAR, and G_s. The finding that $GABA_B$ receptor activation enhances βAR agonist binding suggests that GABA modifies βAR–G_s coupling. This effect could result from an alteration in a region of the βAR that is common to G_s-coupled receptors and is involved in receptor–G_s coupling or from an effect on G_s itself. Both of these possibilites are consistent with the finding that $GABA_B$ receptor activation enhances the cyclic AMP response to many substances, such as adenosine and vasoactive intestinal peptide, which stimulate G_s-coupled receptors in rat brain slices.

Although the absolute changes in βAR agonist affinity seen in response to $GABA_B$ receptor activation are small, they are potentially biologically relevant. For example, exposure of brain membranes to GTP, which is absolutely required for neurotransmitter-stimulated cyclic AMP production, causes only a two- to threefold shift in βAR agonist affinity (O'Donnell et al., 1984). Moreover, it is possible that $GABA_B$ receptor activation elicits a much larger shift in βAR agonist affinity, a portion of which is lost during the membrane preparation.

The cyclic AMP response observed in cortical slices following incubation with catecholamines and $GABA_B$ receptor agonists and the $GABA_B$ receptor-mediated shift in βAR agonist binding share some common features. In both cases, adrenergic agonist affinity is enhanced (Karbon et al., 1984; Karbon and Enna, 1985). In addition, an intact tissue preparation is required to detect either the cyclic AMP-augmenting response or the shift in βAR agonist binding. These data suggest a causal relationship between changes in βAR agonist binding and the cyclic AMP-augmenting response. In any event, the present findings provide additional evidence supporting a neuromodulatory role for GABA in brain and illustrate the functional importance of receptor–receptor interactions.

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Pharmacological and Biochemical Evidence for the Existence of Multiple $GABA_B$ Receptor Subpopulations in Central Nervous System

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INTRODUCTION

A common feature of many neurotransmitter substances is their ability to interact with multiple receptors. For example, norepinephrine activates both α- and β-adrenoceptors, each of which may be subdivided further on the basis of functional properties and pharmacological selectivity. Similarly, acetylcholine acts upon nicotinic and as many as five different muscarinic receptors. The existence of receptor subtypes allows for a limited number of neurotransmitter and neuromodulatory substances to regulate an extensive array of cellular processes.

Amino acid neurotransmitters such as γ-aminobutyric acid (GABA) and glutamic acid also interact with multiple receptors. For GABA, there appear to be at least two receptor subtypes, $GABA_A$ and $GABA_B$. The $GABA_A$ sites mediate changes in chloride ion conductance, are activated by muscimol and THIP and inhibited by bicuculline and picrotoxin. In contrast, $GABA_B$ receptors are bicuculline- and THIP-insensitive and are steroselectively activated by β-p-chlorophenyl GABA (baclofen). Indeed, as described in the present report, there are now data suggesting a multiplicity of pharmacologically distinct $GABA_B$ receptors.

PROPERTIES OF $GABA_B$ RECEPTORS

The $GABA_B$ receptor was initially proposed when it was found that GABA inhibited neurotransmitter release at peripheral sites such as atria, and attenuated electrically-induced smooth muscle contractions in a bicuculline-

insensitive manner. Moreover, these effects are mimicked by baclofen, but not by THIP. While the physiological relevance of peripheral $GABA_B$ receptors is unclear since most organs do not possess GABAergic neurons, these studies pointed to the possible existence of a population of GABA receptors that differed pharmacologically from the classical GABA binding site. Shortly after their discovery in peripheral tissues, $GABA_B$ receptors were identified in the central nervous system when baclofen was shown to inhibit potassium-stimulated neurotransmitter release from brain slices. Clinically, baclofen is employed as an antispastic agent in the treatment of multiple sclerosis (Table 1). Baclofen has also been reported to display analgesic properties, although it is sedating at doses that significantly elevate the pain threshold.

Both in vivo and in vitro, baclofen displays a number of effects on various organ systems (Table 1). The ability of baclofen to inhibit electrically-induced intestinal smooth muscle contractions is particularly interesting inasmuch as GABA is highly concentrated in enteric neurons that synapse upon acetylcholine-containing cells, regulating acetylcholine release. Both balcofen and GABA inhibit electrically-evoked contractions of isolated guinea pig trachea, and attenuate vagally-mediated bronchoconstriction in anesthetized guinea pigs. These findings suggest that selective $GABA_B$ receptor agents might be useful for treating certain pulmonary or gastrointestinal disorders as well as for modifying central nervous system activity. The variety of effects produced by baclofen raises the question as to whether they are mediated by a single population of receptors, or by pharmacologically distinct subpopulations of $GABA_B$ sites.

MULTIPLICITY OF $GABA_B$ RECEPTORS

Following the identification of $GABA_B$ receptors, $GABA_B$ binding sites were characterized in brain membranes using radioligand binding assays. It was found that [^{3}H]GABA labels both low and high affinity $GABA_B$ sites, and that [^{3}H](+)-baclofen, the pharmacologically active enantiomer, also detects low and high affinity sites. Whereas the ratio of low to high affinity $GABA_B$ binding does not differ substantially among rat brain regions, destruction of the dorsal noradrenergic bundle selectively reduces the number of lower affinity $GABA_B$ binding sites, suggesting kinetically distinct populations of $GABA_B$ receptors.

Additional evidence favoring the existence of multiple $GABA_B$ subpopulations receptors was obtained from studies aimed at determining the effector mechanism(s) associated with these sites (Table 2). In rat brain membranes, baclofen inhibits basal and forskolin-stimulated adenylate cyclase activity, decreasing cyclic AMP formation. Thus, $GABA_B$ receptors appear similar to α_2-adrenergic, muscarinic cholinergic, and adenosine A_1, which reduce adenylate cyclase activity by coupling with G_i, the inhibitory guanine nucleotide binding protein. A distinguishing characteristic of G protein-coupled receptors is that agonist binding affinity is reduced in the presence of GTP, which attaches to a regulatory site on the α-subunit of the G-protein. As would be predicted from the cyclase data, $GABA_B$ receptor binding is attenuated by GTP which reduces the affinity of $GABA_B$ recognition sites for the radioligand.

Additional evidence favoring an association of $GABA_B$ receptors with G_i was provided by the finding that islet activating protein (IAP, pertussis toxin), which prevents receptor-G_i interactions, prevents baclofen from inhibiting adenylate cyclase. Likewise, treatment of brain membranes with activated IAP inhibits $GABA_B$ receptor binding, an effect that is reversed by the addition of purified G_i.

While these results suggest that $GABA_B$ receptors are negatively coupled to adenylate cyclase, studies performed with brain slices suggest that GABA can increase brain cyclic AMP levels (Table 2). Thus, in many rat brain regions, including cerebral cortex, hippocampus, and striatum, baclofen enhances neurotransmitter-stimulated cyclic AMP accumulation while having no effect on second messenger formation itself. The response to baclofen is restricted to the (+) isomer, is mimicked by GABA, and is bicuculline-insensitive (Figure 1). This augmenting response is observed using a variety of agents to stimulate cyclic AMP production, including isoproterenol, norepinephrine, adenosine, 2-chloroadenosine, and vasoactive intestinal peptide. While the precise mechanism responsible for the augmentation is unknown, the presence of extracellular calcium ion appears necessary.

In contrast to its effect on neurotransmitter-stimulated cyclic AMP accumulation, baclofen inhibits forskolin-stimulated cyclic nucleotide accumulation in cerebral cortical slices (Figure 2). Therefore, in the same tissue preparation, baclofen may either enhance or inhibit cyclic AMP accumulation, depending upon the agent used to stimulate production of the second messenger. Interestingly, like $GABA_B$ sites, activation of $_2$-adrenergic

receptors causes inhibition of adenylate cyclase in brain membranes but augments cyclic AMP production in brain slices. Therefore, it is possible that functionally distinct $GABA_B$ receptors are present in brain, just as the existence of subpopulations of α_2-adrenergic receptors has been proposed.

Recently, GABA and baclofen have been reported to inhibit histamine (H_1) and serotonin ($5\text{-}HT_2$) receptor-mediated inositol phosphate accumulation in slices of rat and mouse cerebral cortical slices, respectively (Table 2). Whether this represents a direct effect, or is mediated indirectly as a consequence of changes in cyclic AMP production, is unknown. Nonetheless, these findings provide further evidence supporting a neuromodulatory role for GABA acting through $GABA_B$ receptors and should be considered when evaluating the possible existence of multiple $GABA_B$ receptor subtypes.

Electrophysiological studies of $GABA_B$ receptors support the existence of multiple $GABA_B$ receptor subtypes (Table 2). In cultured embryonic dorsal root ganglion cells, GABA and baclofen elicit a bicuculline-insensitive reduction in the duration of the calcium-dependent action potential by decreasing calcium current. A similar mechanism has been proposed to account for the ability of GABA and baclofen to reduce neurotransmitter release from primary afferent terminals.

When applied to rat hippocampal pyramidal cells, baclofen elicits a postsynaptic hyperpolarizing response due to an increase in potassium conductance. $GABA_B$ receptors located presynaptically in the hippocampus and cerebral cortex also inhibit synaptic transmission. Therefore, it appears that

$GABA_B$ receptors are located both pre- and post-synaptically, and influence both Ca^{++} and K^+ ion conductances.

These data indicate that activation of $GABA_B$ receptors causes a variety of cellular responses. It remains unclear, however, whether these responses are mediated by a single $GABA_B$ receptor entity that has different kinetic properties, is differently localized and coupled to distinct effector mechanisms, or whether there exists pharmacologically and functionally distinct $GABA_B$ receptor subtypes. One way to address this issue is through the uses of receptor-selective antagonists. For example, the discovery that bicuculline selectively blocks GABA-mediated responses was vital in establishing a neurotransmitter role for this amino acid. More recently, in an effort to discover selective $GABA_B$ receptor antagonists, the corresponding phosphonic (phaclofen) and sulfonic acid (2-OH saclofen) derivatives of baclofen were synthesized (Figure 3). Phaclofen antagonizes $GABA_B$ receptor-mediated depression of the ileal twitch response, as well as baclofen-induced reduction of interneuron discharge in spinal cord. In brain, phaclofen selectively inhibits K^+-dependent hyperpolarization elicited by baclofen in thalamic, hippocampal, and dorsolateral septal neurons. Likewise, 2-OH saclofen antagonizes GABA- and baclofen-induced depression of electrically-stimulated smooth muscle contractions. Although the utility of these compounds is limited by their lack of potency (pA_2 = 4-5), these studies have contributed to establishing a physiological role for $GABA_B$ receptors.

Using 2-OH saclofen, efforts were made to determine whether the receptors mediating the inhibitory response to baclofen on adenylate cyclase differed from those responsible for augmenting second messenger accumulation in brain tissue.

The results of these experiments revealed that 2-OH saclofen reduces the potency of baclofen to enhance isoproterenol-stimulated cyclic AMP accumulation, and blocks the adenylate cyclase inhibitory response to baclofen (Figure 5). This finding suggests that 2-OH saclofen is incapable of differentiating between these two receptor responses.

Evidence for the existence of pharmacologically distinct $GABA_B$ receptor subtypes was provided by the finding that, like baclofen, 3-aminopropylphosphonic acid (3-APPA) reduced forskolin-stimulated cyclic AMP accumulation, but unlike baclofen, does not enhance catecholamine-stimulated cyclic AMP production (Table 3). The inhibition of the forskolin response by 3-APPA was not additive with baclofen, consistent with the notion that the two amino acid receptor agonists act at the same site. While these findings suggested that 3-APPA is a selective $GABA_B$ receptor agonist, additional studies revealed that it antagonizes the effect of baclofen on catecholamine-stimulated cyclic AMP accumulation (Figure 6). Interestingly, 3-APPA has been reported to be a partial agonist in the gut as well as in the central nervous system, whereas it behaves as an antagonist in guinea pig airway. These findings suggest that in addition to being functionally distinct, the $GABA_B$ receptors associated with the forskolin and catecholamine effects on cyclic AMP production are pharmacologically discrete. In addition, it appears that $GABA_B$ receptors located in the central nervous system may differ from those located in the periphery.

Various GABA derivatives have been tested for their ability to interact with cyclic AMP-generating systems in rat brain slices (Figure 7). For example, both 2-butyl and 2-decyl GABA inhibit the baclofen augmenting response but,

unlike 2-OH saclofen, 2-decyl GABA has no effect on forskolin-stimulated cyclic AMP accumulation (Figure 8).

While these results appear to support the existence of multiple $GABA_B$ receptor subtypes, it is troubling that the concentrations of these compounds required to activate or inhibit $GABA_B$ sites are quite high. Thus, in the cyclic AMP studies, in general it was necessary to examine concentrations of test compound above 100 μM, enhancing the possibility of observing a non-specific effect. For this reason alternative approaches have been used to discriminate between $GABA_B$ receptor subtypes. For example, inasmuch as IAP blocks baclofen-mediated inhibition of adenylate cyclase in brain membranes, attempts were made to determine if toxin treatment also affects the baclofen augmenting response. Indeed, both intracerebroventricular and intrahippocampal injections of IAP prevent baclofen from inhibiting forskolin-stimulated cyclic AMP accumulation. However, whereas IAP treatment reduces the augmenting response in cortical slices, it fails to alter the augmenting response in hippocampal tissue. While this result may be due to the differences in tissue preparation or other experimental variables, it remains possible that the receptor mechanisms differ in these two brain regions. In any event, the results fail to prove whether IAP-sensitive G proteins mediate both the augmenting and the inhibitory responses to baclofen.

Several approaches have been taken to determine whether the pre- and postsynaptic events elicited by baclofen in CA1 hippocampal pyramidal cells are mediated by the same receptor. Phaclofen blocks postsynaptic events, including baclofen-induced hyperpolarizing response and the slow inhibitory postsynaptic

potential seen following CA1 afferent stimulation. In contrast, suppression of the presynaptic excitatory postsynaptic potential elicited by afferent stimulation is phaclofen-insensitive. Likewise, IAP treatment reduces the postsynaptic, but not the presynaptic, response to baclofen. These data also support the existence of distinct $GABA_B$ receptors in terms of their pharmacological selectivity and effector coupling mechanisms.

SUMMARY AND CONCLUSIONS

Much has been learned about the pharmacological, biochemical and physiological properties of $GABA_B$ receptors. These studies have demonstrated that GABA plays a neuromodulatory role through its interaction with $GABA_B$ receptors, and suggest that the $GABA_B$ receptor system is complex. The available evidence seems to favor the existence of pharmacologically and functionally distinct $GABA_B$ receptor subpopulations. Thus, receptor binding experiments revealed that [^{3}H]GABA binds to both low and high affinity $GABA_B$ sites, biochemical analyses indicate the involvement of $GABA_B$ receptors in a variety of second messenger pathways, and electrophysiological studies have shown that $GABA_B$ receptors are responsible for mediating multiple ion channels at both pre- and postsynaptic sites. The present challenge is to determine whether these observations are interrelated, and how each contributes to the physiological role of $GABA_B$ receptors. To address these issues, it will be necessary to develop more potent and selective $GABA_B$ receptor agonists and antagonists. Recently, 3-aminopropylphosphinic acid has been shown to posses $GABA_B$ agonist-like activity in guinea pig ileum and rat anococcygeus smooth muscle preparations with a potency 5-7 times greater than baclofen. An antagonist with equal or greater

affinity would be a valuable pharmacological tool. Indeed, based on present knowledge, it would appear that modification of $GABA_B$ receptor function may prove useful in the treatment of a variety of disorders, including depression, schizophrenia, anxiety, genitourinary dysfunction, and bronchial asthma. Moreover, because GABA seems to act principally as a neuromodulator at $GABA_B$ receptors, receptor agonists and antagonists for this site might prove to be less toxic than existing agents.

TABLE 1

Central and Peripheral Effects of Baclofen

CENTRALLY-MEDIATED ACTIONS

- antinociceptive
- antispastic
- sedative

EFFECTS ON ORGAN SYSTEMS

- reduces intestinal motility *in vitro*
- inhibits airway smooth muscle contractility *in vitro*
- blocks vagally-mediated bronchoconstriction *in vivo*
- reduces uterine and bladder contractions *in vitro*

TABLE 2

Evidence for Multiple $GABA_B$ Receptor Systems

BINDING

- $[^3H]$GABA and $[^3H]$baclofen label both low and high affinity $GABA_B$ binding sites

BIOCHEMICAL

- inhibits adenylate cyclase activity in brain membranes
- reduces forskolin-stimulated cyclic AMP accumulation in brain slices
- augments neurotransmitter-stimulated cyclic AMP accumulation in brain slices
- attenuates neurotransmitter-stimulated inositol phosphate formation in brain slices

ELECTROPHYSIOLOGICAL

- hyperpolarization resulting from increased K^+ conductance
- reduces voltage-sensitive Ca^{++} conductance

TABLE 3

Effect of 3-APPA on Iso- and Forskolin-Stimulated cAMP Production

Figure 1: Effect of (+)(-)Baclofen on NE-Stimulated cAMP Accumulation

TABLE 1

CENTRAL AND PERIPHERAL EFFECTS OF BACLOFEN

CENTRALLY-MEDIATED ACTIONS

- antinociceptive
- antispastic
- sedative

EFFECTS ON ORGAN SYSTEMS

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- reduces uterine and bladder contractions in vitro

TABLE 2

EVIDENCE FOR MULTIPLE $GABA_B$ RECEPTOR SYSTEMS

BINDING

- [^{3}H] GABA and [^{3}H] baclofen label both low and high affinity $GABA_B$ binding sites

BIOCHEMICAL

- inhibits adenylate cyclase activity in brain membranes
- reduces forskolin-stimulated cyclic AMP accumulation in brain slices
- augments neurotransmitter-stimulated cyclic AMP accumulation in brain slices
- attenuates neurotransmitter-stimulated inositol phosphate formation in brain slices

ELECTROPHYSIOLOGICAL

- hyperpolarization resulting from increased K^+ conductance
- reduces voltage-sensitive Ca^{++} conductance

TABLE 3

Effect of Baclofen and 3-Aminopropylphosphonic Acid on Isoproterenol- and Forskolin-Stimulated Cyclic AMP Accumulation

Treatment	Cyclic AMP Accumulation (% Conversion) Control	+ Baclofen	+ 3-APPA
Untreated	0.28	0.49	0.25
Isoproterenol	0.74	1.62	0.71
Forskolin	4.17	2.67	3.02

FIGURE 1

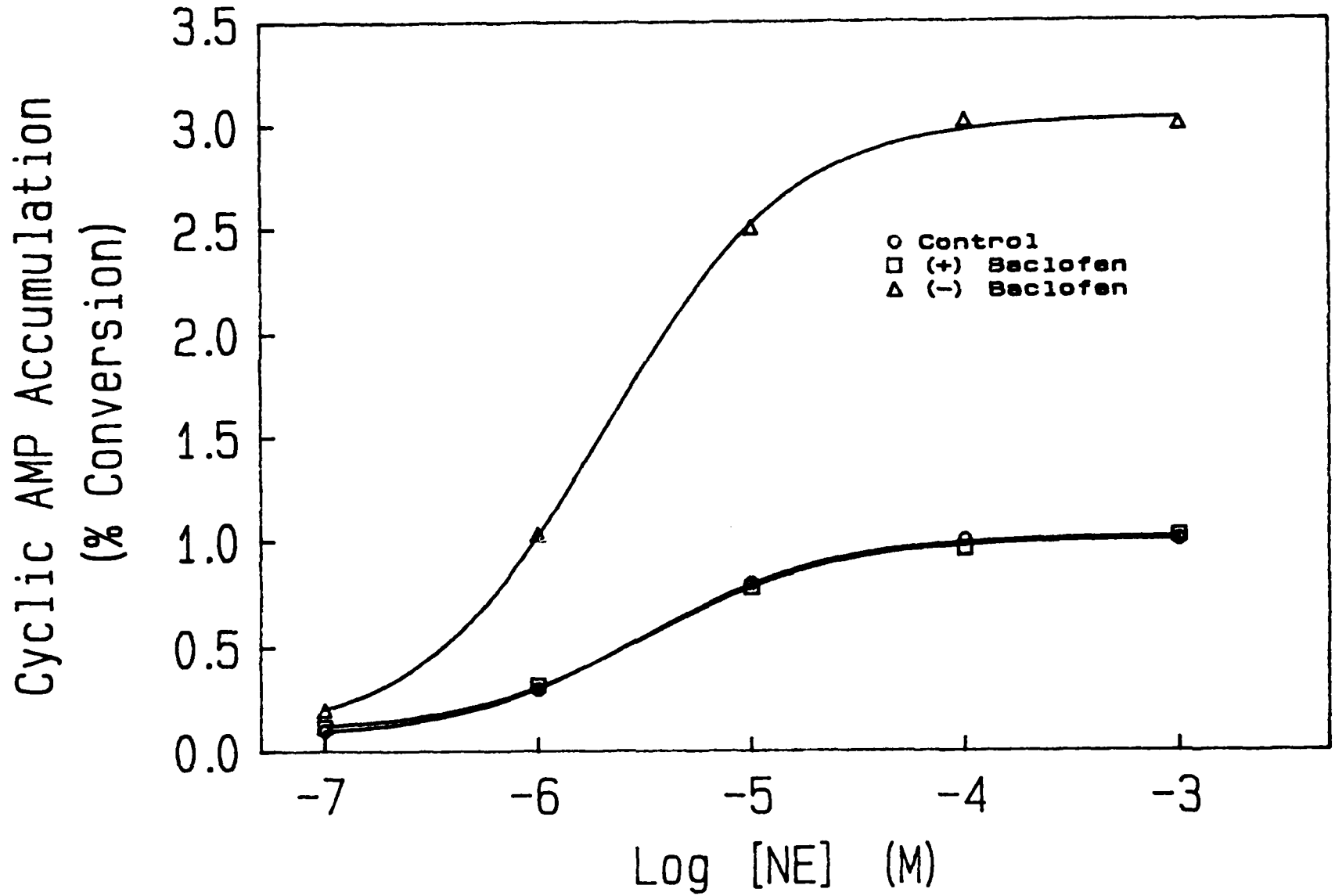

Cyclic AMP Accumulation
(% Conversion)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-7
-6
-5
-4
-3
Log [NE] (M)
Control
(+) Baclofen
(-) Baclofen

Figure 2

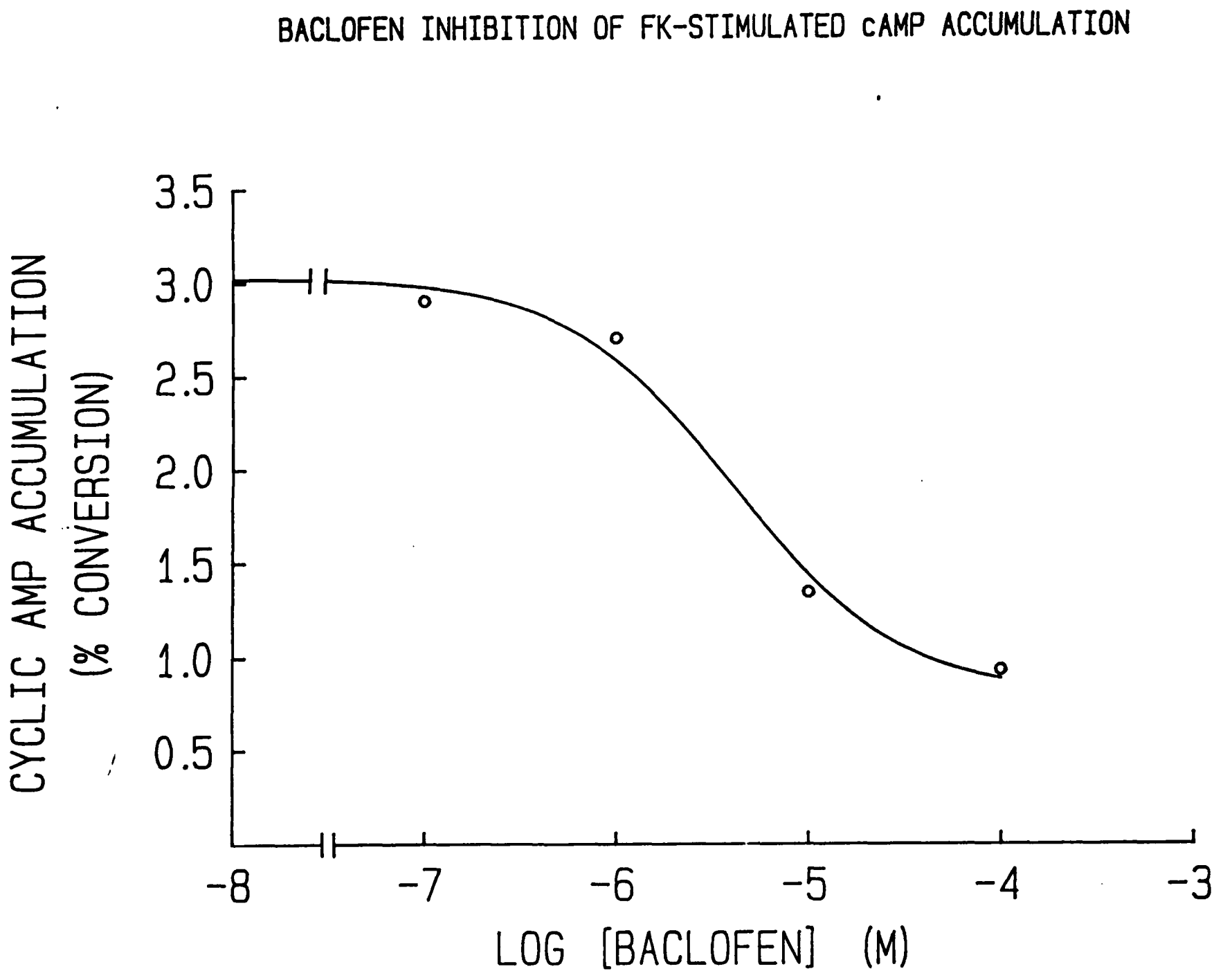
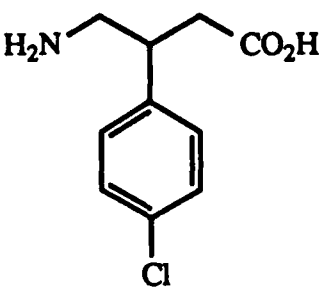
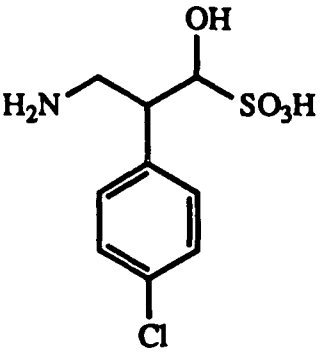

BACLOFEN INHIBITION OF FK-STIMULATED cAMP ACCUMULATION
CYCLIC AMP ACCUMULATION
(% CONVERSION)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
-8
-7
-6
-5
-4
-3
LOG [BACLOFEN] (M)

Figure 3

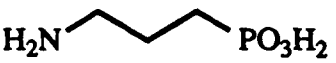


Baclofen

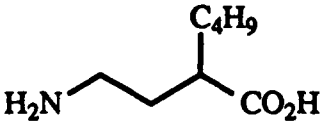


2-OH Saclofen

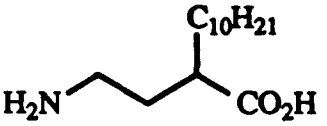
GABA



3-APPA



2-Butyl GABA



2-Decyl GABA

figure 4

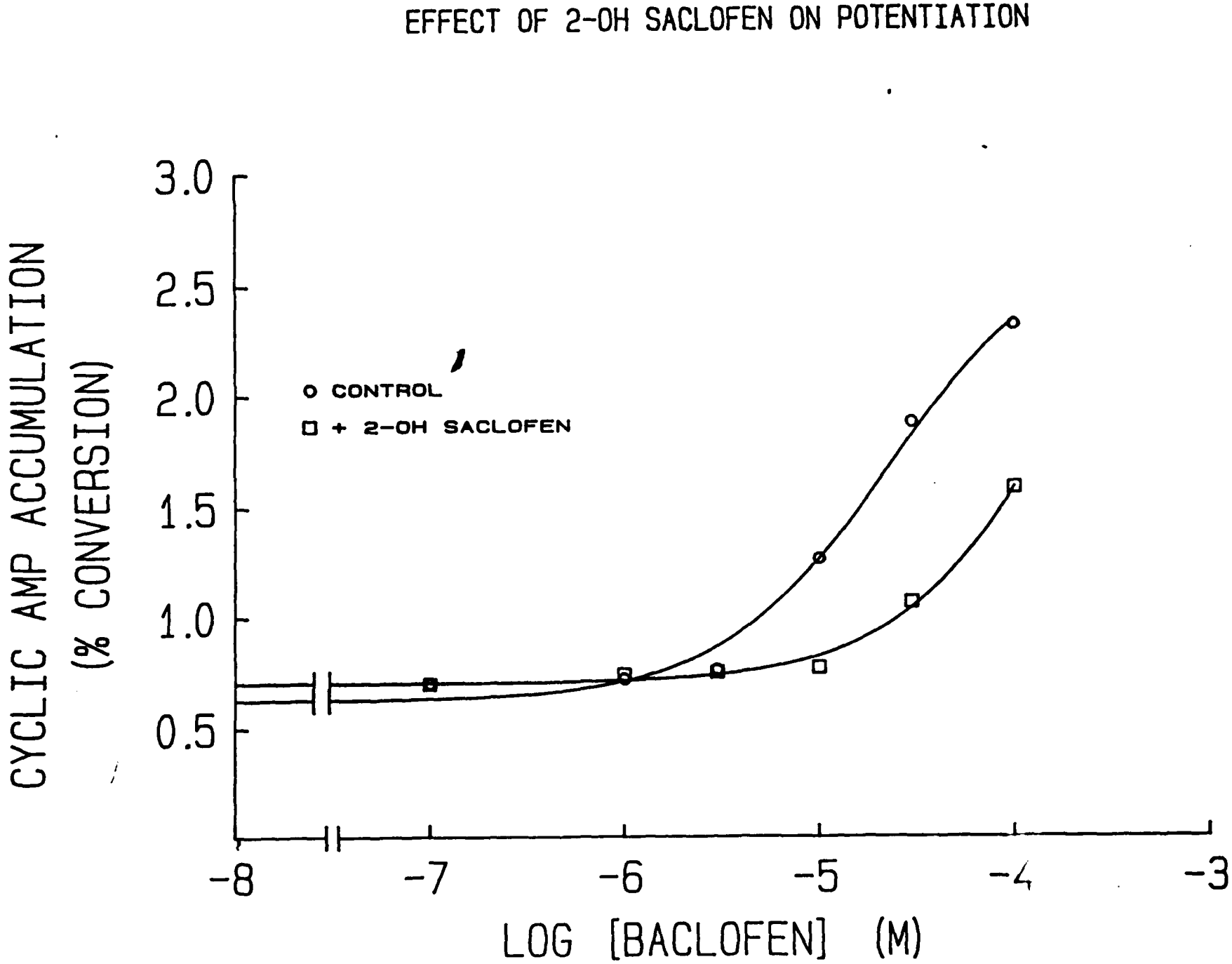

EFFECT OF 2-OH SACLOFEN ON POTENTIATION
CYCLIC AMP ACCUMULATION
(% CONVERSION)
3.0
2.5
2.0
1.5
1.0
0.5
o CONTROL
□ + 2-OH SACLOFEN
-8
-7
-6
-5
-4
-3
LOG [BACLOFEN] (M)

Figure 5.

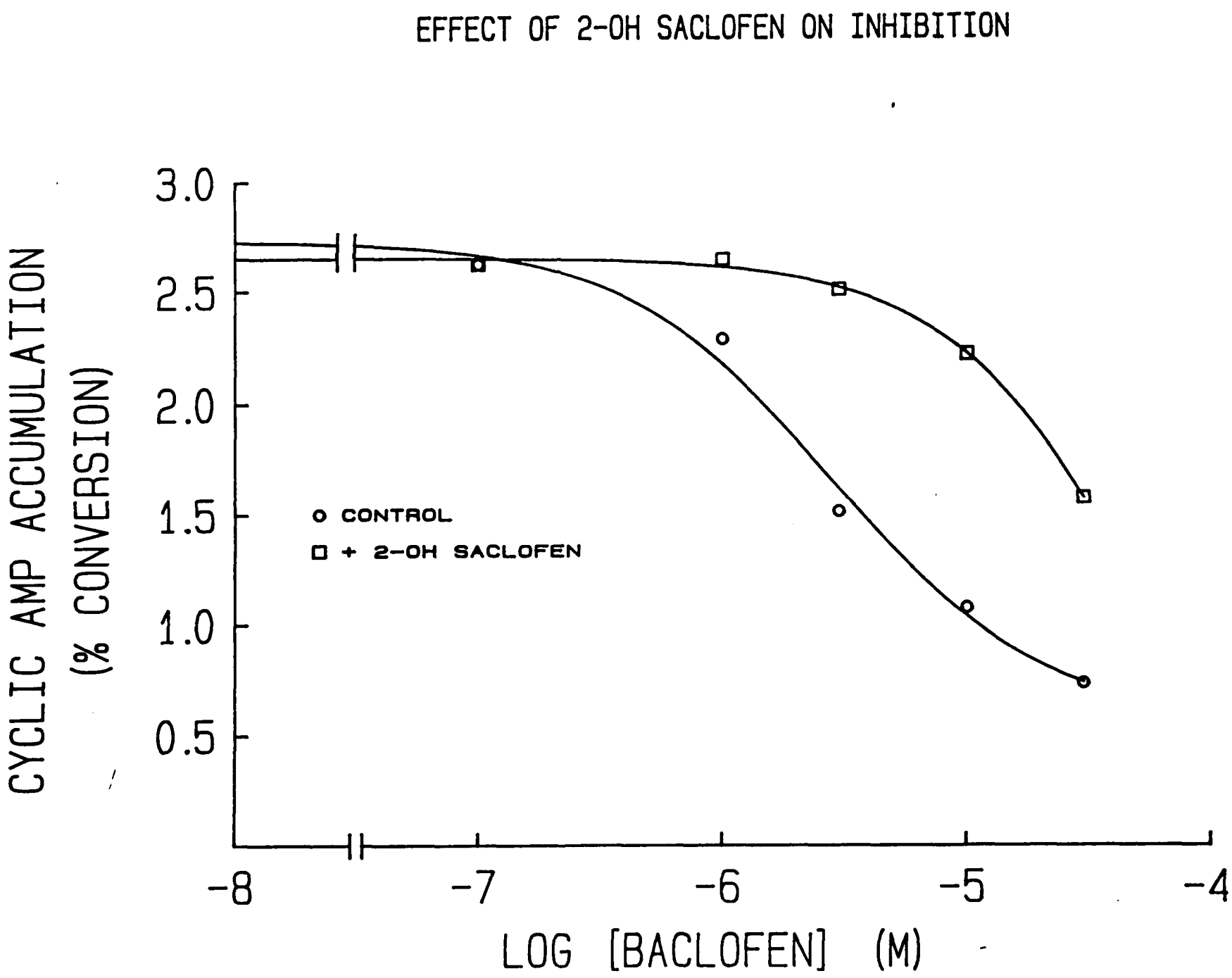

EFFECT OF 2-OH SACLOFEN ON INHIBITION
CYCLIC AMP ACCUMULATION (% CONVERSION)
3.0
2.5
2.0
1.5
1.0
0.5
o CONTROL
□ + 2-OH SACLOFEN
-8
-7
-6
-5
-4
LOG [BACLOFEN] (M)

figure 6.

EFFECT OF 3-APPA ON POTENTIATION

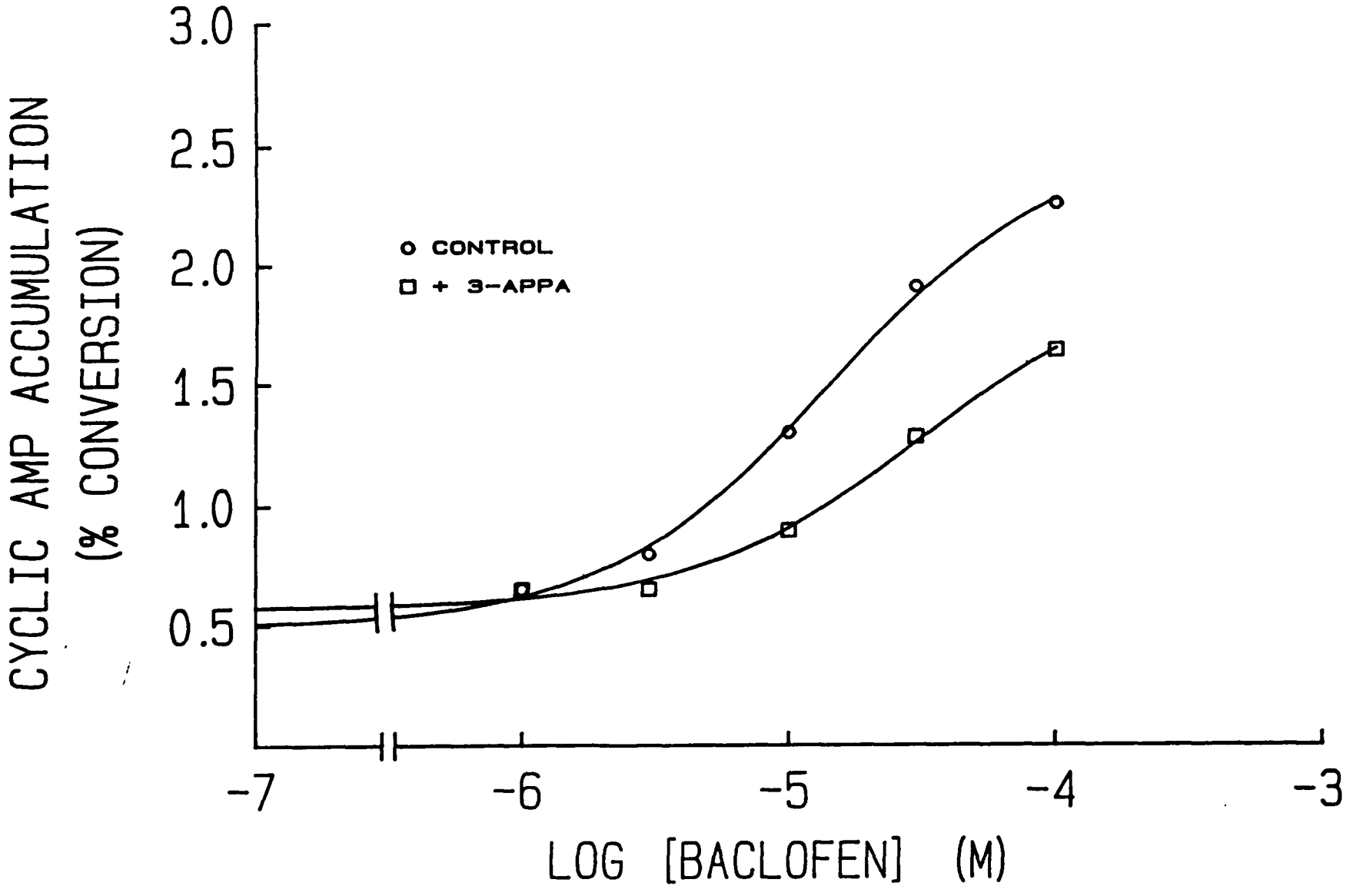


Figure 7

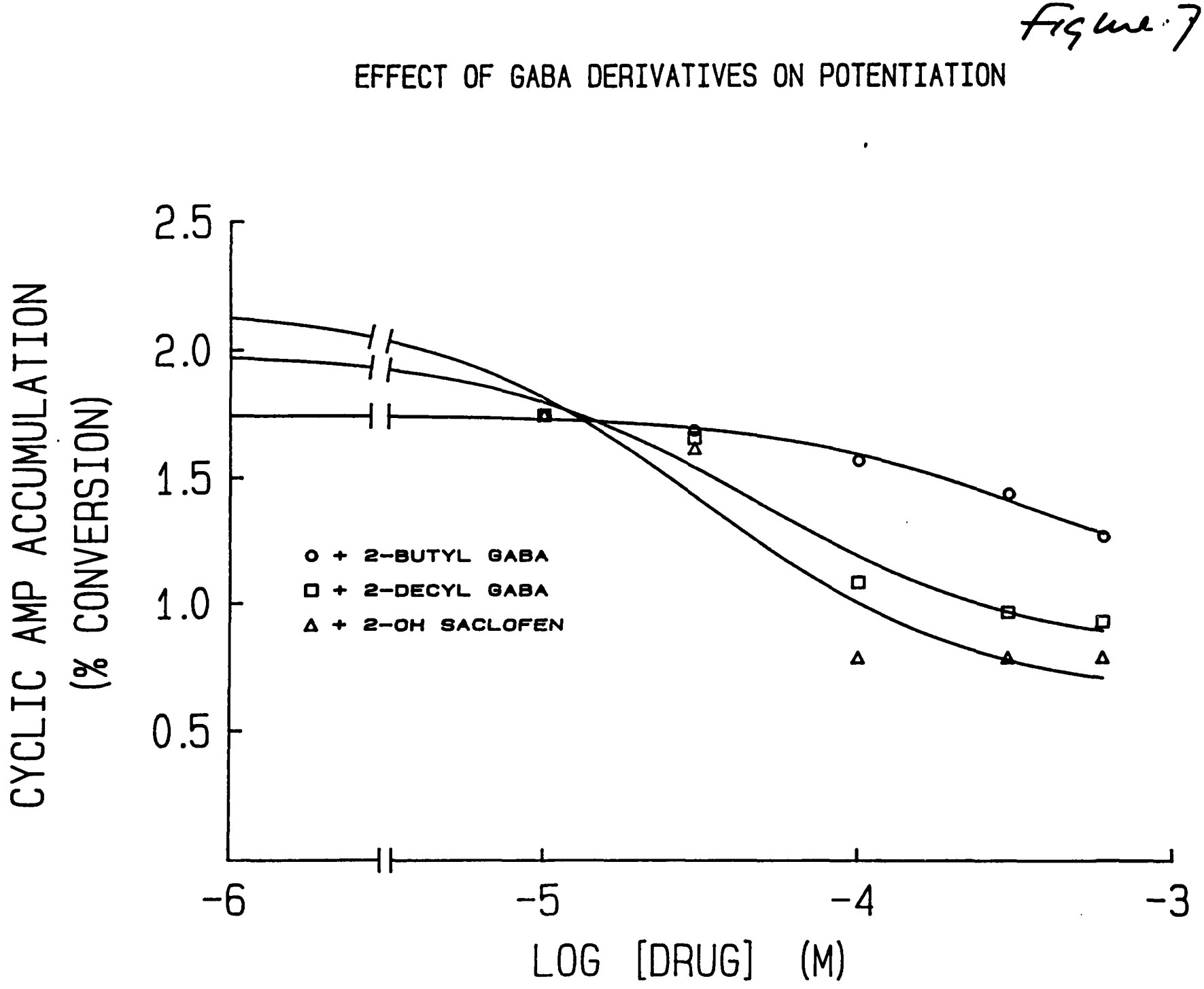

EFFECT OF GABA DERIVATIVES ON POTENTIATION
CYCLIC AMP ACCUMULATION (% CONVERSION)
2.5
2.0
1.5
1.0
0.5
o + 2-BUTYL GABA
□ + 2-DECYL GABA
Δ + 2-OH SACLOFEN
-6
-5
-4
-3
LOG [DRUG] (M)

Figure 8.

EFFECT OF 2-DECYL GABA ON INHIBITION